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Molecular Typing Methods for Pertussis Epidemiology

by Yvon R. de Moissac ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Molecular Typing Methods for Pertussis Epidemiology submitted by Yvon R. de Moissac in partial fulfillment of the requirements for the degree of Master of Science.

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DATE: 13 JANUARY 95

ABSTRACT

The continued presence of the childhood disease whooping cough is due, in part, to difficulties by which the causative organism, *Bordetella pertussis*, is detected. Even more problematic, is that the low culture rate from afflicted individuals makes pertussis a troublesome organism to trace from an epidemiological standpoint. Clinical isolates have been typed serologically for epidemiological purposes, however, this typing scheme has proven too insensitive for tracing *Bordetella pertussis* during outbreak situations. In addition, methods such as multilocus enzyme electrophoresis, restriction fragment length polymorphisms, and ribotyping have not been able to demonstrate adequate diversity between *B. pertussis* strains. Development of better discriminatory tools must be implemented to trace pertussis effectively and in efforts to help control the spread of the organism.

Due to the shortcomings of serology or multilocus enzyme electrophoresis as epidemiological tools, we have explored the use of discriminatory methods based on the further examination of the pertussis genome. One such method is pulsed-field gel electrophoresis (PFGE), and we have shown it to be a highly sensitive tool for pertussis strain analysis during outbreak conditions. Of an initial

random sampling of 28 isolates, 14 from Edmonton and one from each of 14 northern Alberta towns, the resulting DNA patterns were far more heterogeneous than anticipated and caused concern that PFGE was too sensitive. Further analysis showed that this was not the case as clusters of similar PFGE patterns were observed from the same outlying town. Three PFGE types: a, b, and c, predominated in the outbreak, overall accounting for 59 of 79 *B. pertussis* strains tested. Results from isolates from outlying towns, however, indicated involvement of local strains rather than a single, highly infectious strain in the whooping cough outbreak in Alberta.

A major pitfall of a PFGE based epidemiological typing system is that it is very expensive and time consuming. Compared to the seven days it would take to analyse 20 strains, a modified Polymerase Chain Reaction (PCR) technique: random amplified polymorphic DNA (RAPD), can analyze 50 strains at one third of the cost. Using PFGE typed clinical isolates as reference strains, we have compared the ability of RAPD's to discriminate between pertussis clinical isolates. Although the RAPD technique is fast and cost effective, preliminary data, using universal primers, demonstrate the that RAPD analysis of pertussis strains is unable to discriminate between our predominant

PFGE types a, b and c. There may be a possibility that, with a suitable primer, RAPD's may be an alternative to pulsed-field typing of pertussis.

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To my parents, Henri and Léona, it is because of your guidance, love, and support that I am reaching this goal in my life and it is for this reason that I dedicate this thesis to you. Thanks. To my closest friends, Tim and Shane, goes my most sincere gratitude for being in my corner and for teaching me how to enjoy life. You were the best roommates I ever had. Thanks. I also wish to give a bunch o' thanks to all of my friends at Red Robin Longstreet the following of which are especially notable: Christine, Andrea, Shelly's S and T, Wendy, Kym Shae, Janelle, Taneen, Donna, Heather, Bjorn, Ned (Tom) Walker, Derek, Scott, Peter, Dean, Mike, Dan, Joe (Chuck), Guy, Kamal, John, and Kevin. However, my most heart felt thanks goes to my very good friends, Reid Cornell and Drew Megson--you guys are the best. Thanks.

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LIST OF NOMENCLATURE AND ABBREVIATIONS

AGG	agglutinogen
BCIP	5-bromo-4-chloro-3-indoyl phosphate
CHEF	clamped homogeneous electric field
ET	electromorph type
GET	glucose EDTA Tris-HCl buffer
IS	insertion sequence
LM-PCR	ligation mediated polymerase chain reaction
MEE	multilocus enzyme electrophoresis
NBT	p-nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulfate
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer

This thesis is dedicated to my parents, Henri and Léona and to my closest friends: Tim, Shane, Reid and Drew.

"Looking for a place to happen, making stops along the way"

- Gordon Downie

CHAPTER I

INTRODUCTION

A. INTRODUCTION AND RATIONALE FOR EXPERIMENTATION

Members of the genus *Bordetella: pertussis, parapertussis, bronchiseptica,* and *avium;* are small, gram-negative coccobacilli. They are strict aerobes *and B. pertussis* and *parapertussis* require special media enriched with blood and an extremely humid environment (98%) in order to grow. When first discovered in 1906 by Bordet and Gengou (4), *B. pertussis* was classified as a member of the *Haemophilus* genus. It was not until 1940, when Hornibrook (24) discovered neither X nor V factors were required for growth, that he designated the new genus name, *Bordetella.*

Of the four species, only *Bordetella pertussis* and *parapertussis* are human pathogens and are responsible for the disease called whooping cough. The most severe form of the disease occurs in children under the age of one year. The clinical syndrome has three stages. The catarrhal is the primary stage where the organisms colonize the cilia in the respiratory epithelium via filamentous haemagglutinin and pertactin. Symptoms resembling the common cold ensue and last from ten to 14 days. The pharmacological effects of the pertussis and adenylate cyclase toxins become apparent during the secondary or paroxysmal stage. This stage is characterized by a severe cough where a child may suffer from many bouts of paroxysmal coughing each day for a period lasting two or more weeks. In some instances, vomiting may follow coughing spells. The child may also cough to the point of hypoxia until an inspiration of air against a partially closed glottis produces the classical 'whoop' sound. Brain damage may result if the child is hypoxic for extended periods. In the final, convalescent stage, disease severity tapers over a period from two to three weeks to several months. Antibodies to the organism may remain in the bloodstream for many months to years.

Whooping cough is a difficult disease to diagnose and track from an epidemiological standpoint. This is due in part, to the nature of the catarrhal stage, since cold-like symptoms alone are insufficient to raise suspicion of pertussis infection. During this stage the organism may be unknowingly passed on to susceptible individuals in the population. By the time the characteristic paroxysmal stage is reached, there may not be any organisms present to make a culture positive diagnosis. Also, a whoop is not always present (18) and if present, is rarely observed by health care workers responsible for reporting the disease. The result of this is an under reporting of the disease. During a 30 year period, for example, only 5% to 25% of *B. pertussis* infections were reported in England and Wales (10) and under reporting is assumed to occur in the U.S. and Canada (66). In 1993, the Canada Communicable Disease Report published guidelines recommended by the Pertussis Consensus Conference which standardize pertussis case definitions (47).

1. Clinical Case

Cough lasting more than or equal to two weeks and one or more of the following:

a) paroxysmal cough
b) cough ending in apnea or vomiting
c) inspiratory 'whoop'

for which there is no other known cause.

2. Confirmed Case[*]

Either:

laboratory confirmation of *Bordetella pertussis*

---

[*] These criteria have been redefined to exclude the use of the fluorescent antibody as a laboratory confirmation due to the lack of specificity of the test. This was, at one time, one of the confirmatory tests for pertussis (17).

Or:

a clinical case with an epidemiologic link to a laboratory-confirmed case

For the purpose of national surveillance, the preceeding case definitions must be specific to allow comparisons and analysis of epidemiologic characteristics (47). However, for the purpose of outbreak control and prevention, more sensitive definitions must be employed (47).

1. Suspect Case Definition

Any duration of paroxysmal cough, OR coughing with inspiratory whoop, OR cough ending in apnea, vomiting, or gagging for which there is no other known cause.

All physicians should report all suspect cases as soon as possible to permit rapid intervention.

2. Criteria for Public Health Intervention

Either:

Paroxysmal cough or cough with vomiting or gagging greater than or equal to seven days OR cough with apnea OR cough with inspiratory whoop with no other known cause.

Or:

Clinically compatible symptoms and an epidemiologic link with a confirmed case or a setting where there have been cases.

The incidence of whooping cough cycles regularly every three to four years (18). There are two factors suggested to account for the relative stability of this epidemic interval, 1) patterns of immunity and 2) the efficacy of the pertussis vaccine (18). Firstly, patterns of immunity are largely determined by the number of susceptible individuals in the population. The birth rate and immune status of the population both determine the number of susceptibles in the population (18). Since 1943, the distribution of the pertussis vaccine has had a profound impact on immune status of the population. This accounts for a 93% reduction in cases per 100,000 capita observed during the postvaccine period of 1982 to 1991 when compared to cases per 100,000 capita during the prevaccine period of 1932 to 1941 (47). Secondly, vaccination should protect more than those immunized, in theory, because of the indirect protection conferred upon the non-immunized population, i.e. 'herd immunity' (18). Such increased protection from disease should lengthen the cycling period because of the overall decrease in the number of newborn susceptible individuals. However, the efficacy of pertussis vaccines has not been able to affect the cycle of pertussis infection despite the fact that the long term incidence of whooping cough has fallen since the advent of vaccination programmes. This was evident when vaccination compliance dropped dramatically in England and Wales in the mid-1970's but did not result in a subsequent shortening of the cycle (18). Since efficacy and more importantly, the coverage and distribution of the pertussis vaccine determines the amount of protection conferred to the population (18), the possibility of attaining 'herd immunity' is difficult. Presently, vaccination is focused upon young infants to help protect them from disease as well as to prevent the spread of infection. However, it has been observed that adults can carry *B. pertussis* with only minor or no clinical symptoms (40). This population of asymptomatic carriers are capable of passing the infection along to the smaller, unprotected population, that is, the population that should be theoretically protected because the majority have been vaccinated.

In consideration of these difficulties, effective epidemiological methods are a priority to help determine the existence of and trace the movement of virulent strains (47). To this end, there are relevant questions that may be addressed such as: are prevalent strains prevalent because they are more virulent? If so, does this translate to vaccine failure? Could vaccine failure be the result of shifts in antigens produced by disease isolates? The answers to these questions lie within the determination and collection of epidemiologically related clinical isolates, a task that will be made easier by the use of effective epidemiological tools.

B. CONVENTIONAL METHODS FOR PERTUSSIS EPIDEMIOLOGY

1. Serology

There currently is no standardized, sensitive technique for discriminating among strains of *Bordetella pertussis* and *Bordetella parapertussis*. Serological classification of these bacteria is too insensitive for epidemiological studies because of the relative lack in diversity of antigenic markers (lipooligosaccharide, agglutinogens). Unlike most gram negative pathogens, *B. pertussis* has almost no antigenic diversity in its lipooligosaccharide that would constitute the equivalent of heat stable "O antigen" serotypes (30, 32). And since *B. pertussis* and *B. parapertussis* are non-flagellated, there are no "H antigens". Two fimbrial antigens (Fim 2 and Fim 3) constitute the major antigens by which some strains can be distinguished from one another by agglutination (52). Because these antigens stimulate the production of agglutinating antibodies, they have been called "agglutinogens" or "AGGs" (52). Four other agglutinogens, 1, 4, 5, and 6 have been described (15) but their molecular identity is presently unknown, as is their value for epidemiology (51). In practice, AGG 1 is found on all virulent strains of *B. pertussis* and only three serotypes are found, those possessing AGG 1 and AGG 2, AGG 1 and AGG 3, or AGG 1 and both AGG 2 and AGG 3. The epidemiological utility of the AGG types was demonstrated by Preston et al. who observed an antigenic shift in the general *B. pertussis* population from serotypes 1,2,0 and 1,2,3 in 1958 to serotype 1,0,3 in 1963-1964, a change which had a negative impact on vaccine efficacy (48, 49).

Thus the agglutinogens 2 and 3 have proven useful for long-term monitoring of *B. pertussis* strains. But for short-term strain comparisons, they do not provide enough antigenic heterogeneity. Moreover, the expression of AGG 2 and AGG 3 appears to vary in vitro (8, 63) and in vivo (50). These phenotypic changes in surface antigens may be important for pathogenesis but are problematic when using serology for strain characterization.

C. MOLECULAR GENETIC METHODS FOR PERTUSSIS EPIDEMIOLOGY

1. Multilocus Enzyme Electrophoresis (MEE)

Multilocus enzyme electrophoresis (MEE) is a technique by which water-soluble enzymes are resolved based on their net electrostatic charge (57). Since the net electrostatic charge of a protein is based on its amino acid sequence, and therefore, its DNA sequence, mobility variants can be equated to variability within the genetic sequence at a particular locus (57). MEE has been used to study genetic variation in many bacterial genera (11, 12, 55, 58, 69, 71), including the genus *Bordetella* (42). But like serology, little MEE diversity exists in *B. pertussis* or *B. parapertussis* by which interstrain distinctions could be made. Twenty-three strains of *B. pertussis* could be assigned to only three electromorph types and one of these types was unique for the strain used for the intracerebral challenge assay in mice (42). All 21 strains of *B. parapertussis* had identical MEE profiles. In contrast, *Legionella pneumophila* serotype b has 50 MEE profiles (58).

2. Insertion Sequence Typing / RFLP

Bacterial insertion sequences are insertions of the same few segments of DNA throughout a bacterial chromosome (19). They range in size from 800 bp to 2,500 bp (19) and are present in a variety of microorganisms (3, 17, 29, 34, 61), including *Bordetella* spp. (37, 38, 39, 40, 67). In some cases insertion sequences have been observed to be randomly distributed throughout the chromosome. Because of this, they have been targeted for molecular typing strategies. For example, *Mycobacterium tuberculosis* has the insertion sequence IS6110 present in a range of one to 25 copies in its chromosome (62). A labelled *Bam* H1-*Sal* I fragment of IS6110 can be used to probe Southern blots of restriction digested chromosomal DNA to successfully detect restriction fragment length polymorphisms (RFLP) due to the random distribution of IS6110 (35).

Bordetella pertussis also has an insertion sequence, IS481, which is 1,046 bp long and present in about 50 to 100 copies throughout the chromosome (38). However, when a labelled probe to IS481 is applied to Southern blots of restriction digested chromosomal DNA, RFLP's are not detected, that is, the patterns from different strains look the same (21). This has also been confirmed by our own experiments. The reason for the absence of diversity lies within the very conserved nature of the pertussis chromosome and that many copies of IS481 are arranged in tandem rather than assuming a random distribution. Recently, however, another insertion sequence, IS1002, has been discovered in the *B. pertussis* chromosome. Present in only seven to 12 copies, it is randomly distributed throughout the genome. When a probe to IS1002 has been hybridized to *Sma* I restriction digests, diversity between clinical isolates has been observed (41).

3. Ribotyping

Ribotyping is very similar to insertion sequence analysis (RFLP analysis) except that the probes involved are those for ribosomal RNA (rRNA) genes. These probes, which are conserved 16S and 23S rRNA from *E. coli*, are hybridized to restriction digests of chromosomal DNA (65) and RFLP's in chromosomal genes encoding rRNA can be used to show diversity within related species from several bacterial genera (13, 22, 23, 28, 46, 54, 59). Recently, ribotyping has been attempted with clinical isolates of *B. pertussis*. Of 42 strains analysed, 41 had and identical ribotype. The sole deviant was a laboratory strain (45).

4. Pulsed-Field Gel Electrophoresis (PFGE)

The development of pulsed-field gel electrophoresis (PFGE) has been important to the resolution of large molecular weight DNA molecules. Pulsed-field gel electrophoresis, or more specifically, clamped-homogeneous field electrophoresis (CHEF), is able to resolve DNA fragments in the range of 40 kb to 1,000 kb or more by switching the electrical fields from electrodes oriented in a hexagonal array. PFGE has made the mapping of mammalian and bacterial genomes less tedious, and genomic maps for several bacterial species (9, 53, 60), including *B. pertussis* (64) have been constructed using this technique. CHEF has also been used to resolve macrorestriction digests of chromosomal DNA for epidemiological purposes. Again, PFGE typing of bacteria has been applied to many species (2, 7, 21, 25, 56, 61, 70), including *Bordetella pertussis* (14, 26, 27), *B. parapertussis* and *B. bronchiseptica* (27). The use of PFGE has become a very sensitive method for epidemiologic strain analysis in the genus *Bordetella*. Its major drawbacks, however, is that it is a very time consuming and tedious technique. The average time for the genotypic analysis of twenty strains from culture to a DNA type is five to six days.

5. Polymerase Chain Reaction (PCR)

a) Ligation-Mediated Polymerase Chain Reaction

The ligation-mediated polymerase chain reaction is a modification of PCR (44). Chromosomal DNA is first digested with a restriction endonuclease, then a synthetic, compatible linker is ligated to the sticky ends of the restriction fragments. Two primers for PCR are then required: the first is specific to the synthetic linker, while the second is outward facing from an insertion sequence. The result is the amplification of regions from a restriction site to the edge of an insertion sequence--in effect an amplified restriction fragment length polymorphism. This technique has been used to successfully type *M. tuberculosis* isolates (44) as an efficient alternative to conventional RFLP analysis.

b) Random Amplified Polymorphic DNA (RAPD)

This variation of the polymerase chain reaction involves a drastic decrease in temperature (36°C as compared to 60°C in 'standard' PCR techniques) during the annealing stage of the reaction cycle to allow short (10 to 12 bp), random oligonucleotides to anneal. Based on genomic variation, the oligonucleotides were initially shown to reproducibly amplify DNA from eukaryotic, plant, and prokaryotic species (69). Since these initial experiments, RAPD's have been increasingly used to successfully genotype many other bacterial species (1, 5, 6, 36, 43, 63). In the case of *Candida albicans* (5), the level of discrimination of RAPD analysis is almost that of PFGE.

D. DISCUSSION

With the number of techniques established for bacterial strain typing, it is the main objective of this thesis to apply one or more of them to the epidemiological study of *Bordetella pertussis*. Due to the shortcomings of serology, multilocus enzyme electrophoresis, and RFLP analysis, the use of pulsed-field gel electrophoresis was the first technique to be attempted in order to reach our objective. The result of these attempts was the development of a sensitive technique for *B. pertussis* strain analysis which could be used during outbreaks (14). The only drawback to the success of PFGE for *B. pertussis* typing was that it is time consuming and expensive when one considers the other techniques available.

Thus, using PFGE as a 'reference' molecular genetic typing method of *B. pertussis*, other techniques, namely established PCR strategies that could reduce sample processing and analysis time to a minimum, were explored as alternatives. Firstly, ligation-mediated PCR was attempted to accelerate the generation of typing data. However, due to the conserved nature of IS481 (20), this was not feasible. Efforts to modify LM-PCR such that amplification would occur between an infrequent and a frequent restriction endonuclease to which respective synthetic, compatible linkers were ligated, also met with limited success. With the discovery of the randomly dispersed IS1002 (41), further attempts were made, but were also unsuccessful, most probably due to the infrequency of IS1002 in the pertussis chromosome. Secondly, at the current stage of research, the RAPD technique does not look promising. Certain oligonucleotides, able to show differences in *Pseudomonas* and *Mycobacteria* (31), can generate complex RAPD patterns in pertussis, however, these patterns are identical for the pertussis type a, b and c strains. Furthermore, RAPD patterns on nine pertussis strains were identical when one of these primers was used. More primers have yet to be screened, however due to time constraints, the research could not be completed.

In May 1993, the Pertussis Consensus Conference placed development of new methods for pertussis epidemiology as a high priority (47). The results in this thesis will contribute to this priority by establishing one sensitive molecular genetic technique: pulsed-field gel electrophoresis. This work will be continued in order to find faster, less expensive alternative such as the RAPD PCR method.

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CHAPTER II

Use of Pulsed-Field Gel Electrophoresis for Epidemiological Study of *Bordetella pertussis* in a Whooping Cough Outbreak.[1]

A. INTRODUCTION

Pulsed-field gel electrophoresis (PFGE) has become a useful tool in the study of bacterial genomes. Analysis of DNA fragments generated by rare-cutting restriction endonucleases can be resolved by PFGE allowing the accurate determination of genome sizes as well as genome maps for *Escherichia coli* K-12 (14), *Pseudomonas aeruginosa* (11), *Campylobacter jejuni* (3), and *Bordetella pertussis* (18). Resolution of restriction endonuclease cleaved DNA by PFGE has also been used as a "fingerprint" for epidemiological differentiation of *Campylobacter* sp. (22), *Legionella pneumophila* (13), *E. coli* (1), enteroinvasive *E. coli* (5), methicillin resistant *Staphylococcus aureus* (7), *Listeria monocytogenes* (2), *Shigella* sp. (16) and *Bordetella* species (8,9).

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[1] A portion of this chapter has been published. **de Moissac, Y. R., S. L. Ronald, and M. S. Peppler.** 1994. J. Clin. Microbiol. **32**: 398-402.

The aim of the present study was to test PFGE as an epidemiological tool for classifying clinical isolates collected during a large whooping cough outbreak in Alberta, Canada that occurred from 1989-1991 (4).

Khattak et al. (8, 9) have shown PFGE to be an effective technique for the epidemiological study of *B. pertussis*. These authors showed genotypic diversity in isolates from Germany and the United Kingdom. Because there are only three serotypes, it was not surprising that they could not show any genotype-serotype correlations. Their typing scheme was based on *Xba* I digestion of *B. pertussis* DNA which generated eight to eleven DNA fragments per isolate. The mobility of fragments in the range of 200 to 412 kb showed diversity which resulted in the assignment of 17 PFGE types for the 105 isolates tested. The amount of diversity varied from a six band difference between DNA types 6 and 4 to a one band difference between DNA types 9 and 12. The authors suggested that PFGE typing could be used to track the movement of *B. pertussis* strains within and/or between towns, communities, and cities (9).

During the period of December 1989 to May 1991, the province of Alberta experienced its largest recorded whooping cough outbreak since the beginning of vaccination in 1943 (4). This event provided a unique opportunity to test new epidemiological tools. Our first attempts at PFGE, which were conducted at the same time as Khattak et al., convinced us that this technique was able to distinguish between different laboratory strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. At the same time it showed that isogenic phase variants of *B. pertussis* produced identical patterns. Based on these results, we conducted a preliminary study by randomly selecting isolates from towns and cities spanning northern Alberta to get an impression of the genetic diversity shown by the clinical isolates. Following the preliminary survey, we examined two small outbreaks. In total, 79 strains were examined of the 552 collected during the outbreak period. Finding identical PFGE types in different geographical location suggests the movement of strains from one town to another. Our data suggest that the outbreak in northern Alberta was probably a result of immune status in the population rather than as a result of a particularly virulent strain.

B. MATERIALS AND METHODS

1. Bacterial strains and enzymes.

The laboratory strains of *Bordetella* species used in this study are listed in Table II.1. The clinical isolates were provided by the Provincial Laboratory of Northern Alberta, (Edmonton, Alberta, Canada). Organisms were cultured on Bordet-Gengou agar (Difco, Detroit, MI) using 15% defibrinated sheep's blood (Triage Labs, Ardrossan, AB) and incubated at 37°C in 98% humidity. The restriction enzymes *Hpa* I (5′-GTT/AAC-3′) and *Spe* I (5′-A/CTAGT-3′) were obtained by Sigma Chemical Co., St. Louis, MO. The restriction enzyme *Xba* I (5′-T/CTAGA-3′) was obtained by Boehringer Mannheim Canada, Laval, Québec.

2. Preparation of agarose embedded DNA.

Chromosomal DNA was embedded in low melting point agarose to prepare DNA plugs for subsequent restriction endonuclease digestion and analysis by PFGE as described by Smith et al. (25). Three to four day old cultures were suspended in 5 mL of 50 mM Tris-HCl, 5mM EDTA, pH 8.0, (1X TE buffer). Suspensions were standardized to A_{540} of 0.80 in 13 x 100 mm screw-capped tubes and transferred to a 25 mL Corex centrifuge tube. An extra 5 mL of 1X TE was added to the suspension and the cells were centrifuged at 4°C for 10 minutes (8000 x g) in 1X TE. Pellets were washed twice more, then resuspended in 2.5 mL of 10 mM $MgCl_2$ and were mixed with 2.5 mL of low melting point agarose (Chromorose, Clontech, Missisauga, Ontario). Two percent low melting point agarose was prepared in 0.5X Tris-Borate-EDTA (0.9 M Tris-borate, 0.02 M EDTA; 5 X TBE). The bacteria-agarose mixture was gently but quickly mixed and carefully poured onto a clean, level, glass microscope slide and allowed to solidify at room temperature for about 15 minutes. A gel lane cutter (BioRad, Missasauga, Ontario, cat.# 170-4120) or a large razor blade was used to cut the solidified mixture into plugs of approximately 6 x 4 x 2 mm. The plugs were transferred to a 15 mL tube and exposed to 10 mL of a lysis buffer consisting of 0.1 M EDTA (pH 8.0), 0.01 M Tris-HCl (pH 7.6), 0.5% sodium dodecyl sulfate (SDS), 0.5% lauroyl sarcosine, 0.2% deoxycholic acid, 200 µg/mL RNAse, and 1 mg/mL lysozyme and was incubated at 37°C for 16 to 18 hours. After incubation, the lysis buffer was removed and replaced with 10 mL of a proteinase K solution (1% lauroyl sarcosine, 1 mg/mL proteinase K (Boehringer Mannheim), 0.5 M EDTA) and incubated at 50°C for eight hours and then 37°C overnight. Following incubation, the plugs were washed for two, one-hour intervals at 37°C in 1X TE, two half-hour intervals at room temperature in 1X TE with 1 mM phenylmethylsulfonylfluoride (PMSF), and two half-hour intervals at room temperature in 1X TE. The plugs were stored in 1X TE at 4°C.

3. Digestion by restriction endonuclease.

One DNA plug prepared as outlined above represents an approximate volume of 40 µL. A digestion mixture of a final volume of 200 µL consisted of: one DNA plug, 136 µL of sterile, distilled, water, 20 µL of the supplied 10X enzyme buffer, 2 µL of 100 mg/mL BSA, and 30 Units of restriction endonuclease. The reaction mixture was incubated overnight at 37°C and stored in 1X TE at 4°C until the plug could be loaded onto a gel.

4. Pulsed-Field Gel Electrophoresis.

The PFGE apparatus used in this study used contour-clamped homogeneous electric field electrophoresis (CHEF). It consisted of a 2015 Pulsaphor Plus Control Unit powered by an electrophoresis power supply EPS 500/400 and cooled by a 2219 Multitemp II Thermostatic Circulator (Pharmacia Biotech, Inc., Baie d'Urfe, Québec). Digested DNA plugs for

CHEF resolution were loaded onto a gel consisting of 1% KILOrose agarose (Clontech, Missasauga, Ontario) in 0.5 X TBE. Approximately one third to one half of the digested plug volume was loaded. Pulse times were programmed into the CHEF apparatus to give a total run time of 24 hours. The pulse times are ramped over six phases beginning at 15 seconds for the first phase and increasing by five second increments for each new phase. Each of the first three phases were run for three hours and each of the last three phases were run for five hours at 175 V at 8°C. These conditions consistently resolved DNA fragments in the range of 50 to 534 kb based on the DNA standards (Lambda Ladder PFG Marker, New England Biolabs, Missisauga, Ontario). The gels were stained in 0.5 μg/mL ethidium bromide for 20 minutes and visualized by a UV transilluminator. The DNA bands were sized by comparison of migration distances to those of the lambda ladder DNA standard.

C. RESULTS

1. Chromosomal digestion of Bordetellae by restriction endonucleases.

Many restriction enzymes were screened for their ability to cut Bordetella chromosomal DNA into small numbers of large fragments resolvable by PFGE and like others (9, 18) we found that *Xba* I and *Spe* I are suitable for this purpose. We have also observed that *Hpa* I is also suitable. *Xba* I was used in this study because it was the most economical, and our results could be compared with those of Khattak et al. (8, 9) and Stibitz and Garletts (18). For the purpose of strain typing, uppercase letters are used for laboratory strains while lowercase letters are used for clinical isolates. There is no special relationship implied between uppercase and lowercase PFGE types (e.g.: B and b).

2. Analysis of laboratory strains by PFGE.

We first tested the fidelity of PFGE by analyzing different laboratory strains of *Bordetella spp.* digested by *Xba* I (Fig. II.1). Between 12 and 16 fragments in the molecular weight range of 50 to 534 kb were produced by *Xba* I digestion of the seven *B. pertussis* laboratory strains (lanes 1 - 7). Digestion of *B. parapertussis* and *B. bronchiseptica* chromosomes by *Xba* I produced 18 and 23 fragments in the same molecular weight range respectively (lanes 8 and 9). The chromosomes of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were completely digested by *Xba* I.

Of the *B. pertussis* laboratory strains, both Tohama I and Maeno strains share the same DNA restriction profile which we assigned to a specific PFGE type (type A). It is different than the profile of the phase III Sakairi strain (type D) isolated from the same outbreak in Japan in 1953 (19). The other *B. pertussis* strains (3779, 2753, and 11615) all have unique DNA profiles, and hence have different PFGE types (types B, C, and D respectively). Only one laboratory strain, 134, shares PFGE type b with some of the clinical isolates. Isogenic phase variants of the same *B. pertussis* laboratory strains were also analysed and it was observed that vir$^+$ and vir$^-$ phase variants of a single strain shared the same PFGE type (data not shown).

3. Analysis of clinical isolates by PFGE.

Our survey of clinical isolates was based on the analysis of 28 strains: 14 from the city of Edmonton, and one from each of 14 northern Alberta towns. Between eight and nine fragments in the molecular weight range of 130 kb to 534 kb were generated by *Xba* I digestion which resulted in a diverse array of DNA profiles. Bands less than 130 kb were not resolved adequately to provide useful information. Of the 14 Edmonton isolates, 10 unique PFGE types were observed. Differences in the 10 unique DNA profiles ranged from a low of one band between types b and o and between types c and g, to a high of five between types h and c (Fig. II.2). In addition, six common restriction fragments were observed in the Edmonton isolates. Fragments with molecular weights of 300 kb, 190 kb, and 130 kb were seen in all 14 isolates while fragments of molecular weights 260 kb, 200 kb, were seen in 13 of the 14 Edmonton isolates. The 150 kb fragment was seen in 12 of the 14 Edmonton isolates.

Xba I digestion of the 14 northern Alberta isolates resulted in seven to ten fragments in the 130 kb to 534 kb range. In contrast to the Edmonton isolates, the northern Alberta isolates had only six different PFGE types among them (a, b, c, d, e, and g Fig. II.2). Of these, four (a, b, c, and e) were identical to five of the 10 Edmonton PFGE types. The remaining two unique patterns increased the total number of clinical PFGE types from 10 to 12.

Differences in the DNA profiles of the Northern Alberta isolates were essentially the same as those observed with the Edmonton isolates. The same common fragments were also observed.

Additional clinical isolates were obtained from siblings to test the ability of PFGE to determine if intra-familial transmission was occurring (Fig. II.3). Of these was a 30 year old mother and her 13 year old daughter from Edmonton (type m), a pair of seven year old twins from Edmonton (type k), a pair of siblings from Berwyn (type b), and a pair of siblings from Manning (type b). Our study also included two different pairs of siblings from the same small town, High Level. These four individuals shared the same DNA profile (type c) suggesting that contact of some fashion occurred between them. Of the six pairs of intra-familial isolates, each individual of a particular pair had an identical PFGE type as their sibling or parent.

Thus, after the analysis of Edmonton, Northern Alberta, and sibling isolates, 40 strains were examined (18 from Edmonton and 22 from Northern Alberta). This resulted in the generation of 13 distinct PFGE types, 11 of which can be found in Edmonton. The fourteenth type was identified in samples analysed from Whitehorse, Yukon.

It was originally thought that, of the 40 aforementioned strains, there were 15 PFGE types (type f being one of the fifteen). However, upon closer examination of 'type f' isolates, it was determined that these isolates were in fact type c strains. This is relevant as the two strains that were once 'type f' were from Edson and Hobbema. Nine other isolates from Hobbema were examined which brings the total number of strains examined to 49.

To further test the usefulness of PFGE for the epidemiology of pertussis, isolates from three smaller, more isolated communities were examined. One was Fort Smith, population 2,480, (#1; Fig. II.6) where 18 culture positive cases occurred within a three month period during the outbreak. The second occured in Whitehorse, The Yukon, population 17,925, where 12 culture positive cases occurred within a 12 month period. Thirdly, ten samples from the native reserve, Hobbema were analysed. The PFGE profiles from all 18 Fort Smith isolates are identical (Fig. II.4) suggesting a single strain (strain a) was responsible for the outbreak in Fort Smith. Type a strains were also isolated from Fort McMurray, Lloydminster, Athabasca, Ponoka, and Edmonton and is a prevalent strain, representing 24 of the 79 (30%) strains analyzed. Eleven of the 12

Whitehorse isolates (Fig. II.5) share a DNA profile (type b) that is also seen distributed across northern Alberta and is seen as far south as Red Deer (#19; Figure II.6). The other type from Whitehorse is type o. Type b strains were prevalent in the outbreak as it represented 24 of the 79 (30%) strains tested. Of the ten of samples from Hobbema (#12; Figure II.6) analysed, four shared a common type, type b. The other 6 strains from Hobbema were types c (3), j (1), and l (2). Type c strains were the next predominant strain, representing 12 of the 79 (15%) strains tested. Thus, the three pertussis PFGE types a, b, and c were observed in 60 of 79 (75%) of the isolates tested so far. Figure II.6 also shows evidence of contact between towns as types a and b and others, such as types e and c, are seen in different locations across the province.

D. DISCUSSION

Our results suggest that PFGE of *Xba* I digested chromosomal DNA provides a sensitive means for discriminating between *Bordetella* isolates at both the species and strain levels. Laboratory strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were easily resolved from each other by major differences in PFGE patterns. It was shown that two *B. pertussis* strains (Tohama I and Maeno) shared an identical DNA profile yet had a profile unique to that of the phase III Sakairi strain. Since these three strains originated from the same outbreak in Japan (19), we were encouraged that resolving *Xba* I digested *B. pertussis* DNA by PFGE could be a useful epidemiological tool. We also analysed laboratory strains of *B. pertussis* which were isogenic phase variants. It was also encouraging to observe that vir$^+$ and vir$^-$ phase variants of a single strain were genotypically identical based on PFGE. These results suggest that frameshift mutations responsible for phase variation as described by Stibitz et al. (17) has no effect on PFGE profiles. During this experiment we also observed how effectively this technique could be used to verify the identity of standard strains. When frozen working suspensions of isogenic strains of vir$^+$ and vir$^-$ Sakairi were used, their DNA profiles did not match, although the profile of vir$^-$ Sakairi perfectly matched the profiles of vir$^+$ and vir$^-$ variants of strain 3779. PFGE of reference suspensions of the same strains confirmed that the vir$^-$ strain of Sakairi had been mislabelled (results not shown). Thus the PFGE pattern types of standard laboratory strains can be used to confirm strain identity in much the same way that karyotyping is used for confirmation of eukaryotic cell identity.

Our preliminary epidemiological data were based on the analysis of 28 clinical isolates of *B. pertussis*, 14 from Edmonton and 14 from towns spanning northern Alberta. We were surprised by the heterogeneity of the DNA profiles exhibited by the isolates: 10 different DNA profiles from 14 Edmonton isolates and a total of 12 different DNA profiles for all 28 strains tested. Because of this, we considered that PFGE may reveal too much heterogeneity in the DNA patterns, so that little or no clustering of strains could be seen (i.e.: a different pattern for each strain). Based on the similarity of profiles from sibling isolates as well as isolates from the small communities of Fort Smith, Hobbema, and Whitehorse, we no longer consider this a problem.

Khattak et al. (8, 9) have already established a PFGE-based genotypic classification system for *B. pertussis* strains, so we sought to match our pattern types with theirs to provide consistency in nomenclature. We have been unable to align our types with theirs. There may be two reasons for this: i) the European strains tested were all unique compared to ours or ii) the electrophoretic conditions used by Khattak et al. make comparison difficult by profile or by conversion to fragment sizes. We believe the latter is the case because their electrophoresis parameters specify a single pulse time of 25 seconds over a 40 hour period at 150 volts whereas our system ramps the pulse times from 15 to 45 seconds over a 24 hour period at 175 volts. In addition, their gels are cast thicker (150 mL of 1% agarose) than ours (100 mL of 1% agarose). Both systems are able to obtain similiar resolution of fragments in the 130 kb to about 300 kb range. Khattak et al. report fragments with molecular weights of 280 kb, 155 kb and 135 kb to be present in all *B. pertussis* isolates they tested. Although our system is different we observed fragments with molecular weights of 300 kb, 150 kb, and 130 kb to be present in virtually all of the *B. pertussis* isolates we tested. However, even using these fragments as a source of reference, correlations between the two systems could not be made. Thus, fragments within this range may or may not differ as a result of true genotypic differences. Problems arise when fragments larger than 280 kb are compared. Because Khattak et al. did not incorporate longer pulse times in their parameters, the resolution between the 280 kb and 412 kb fragments are limited (9). The physical distance between these two fragments is short (about 8 mm) which makes comparisons between the two systems particularly difficult. In contrast, our parameters closely resemble those used by Stibitz and Garletts (18) to physically map the chromosome of Tohama I. Accordingly, our results with *Xba* I digests of strain Tohama I (lane 15, Fig. II.2) are very similar to those obtained by Stibitz and Garletts (18, lane 1, Figure 1a). Efforts to exchange strains for subsequent analysis on respective systems are currently underway to compare our types with those of Khattak et al.

The PFGE results we obtained on clinical isolates were used to address two theories as to the reason for the year-and-a-half long whooping cough outbreak: i) a decrease in the immunized population resulted in an increase in the population susceptible to pertussis disease and ii) the emergence of a highly infectious strain of *B. pertussis* in the province affected both the immunized and non-immunized populations. The latter theory stemmed from the thought that a particularly virulent strain of *B. pertussis* was brought into Canada from outside the country by an individual or a group of individuals attending an International Storyteller's Convention that took place in Whitehorse, Yukon (21). This is why isolates from this particular site outside the province, and over 2,000 km from Edmonton, were included in our study. Although a very prevalent strain of pertussis (type b) was found in Whitehorse, Yukon and as far south as Red Deer, Alberta, over 2,000 km away, we can safely say that type b strains cannot be blamed for the entire outbreak. In particular, type a strains were also widely distributed during the outbreak. Moreover, local strains of other PFGE types besides a and b may be responsible for disease in outlying towns during course of the outbreak. For example, it is possible that type c may be the predominant type in High Level, population 2,849, as two different pairs of siblings share one PFGE type. However, until more isolates from High Level are analyzed, we cannot be sure if close contact actually occured between these four individuals or if a type c strain of *Bordetella pertussis* is responsible for the majority of whooping cough cases in High Level.

Regarding the first theory, the outbreak occurred during the peak of the periodic 4-year cycle of pertussis infection when disease would be expected to be highest. However, 57% of greater than 5600 cases for which records were available had full (at least four doses of vaccine) immunization (6). This suggests that perhaps vaccination was less protective than in previous periods of peak pertussis activity. Isolates from Hobbema were included in this study due to reports of noncompliance with vaccination protocols.

Notable was the heterogeneity of PFGE patterns of Edmonton isolates compared to those from the rest of the province. One possible explanation may be that the city of Edmonton, population 616,741, is visited by many travellers who may bring in different strains. This would allow many different strains to become established and spread throughout the city. Despite this heterogeneity, there is evidence of close contact transmission as shown on Figure II.3, lanes 1, 2, 5, 6. As more isolates from different areas of the city are tested, it would be interesting to see if certain PFGE types predominated in different localities within the city.

With PFGE data such as those generated in the present study, these aspects of whooping cough epidemiology may be addressed. In particular, the immune response to vaccine strains can be compared to the immune responses of children convalescing from the predominant strains in an outbreak. The responses to similar antigens can be contrasted to see if Bordetella organisms have changed, even subtly, so that vaccination failure is more common now.

Pulsed-field gel electrophoresis of *Xba* I digested Bordetella DNA has the potential to be a better method than serotyping based on agglutinogens (9) or multilocus gel electrophoresis (10) for differentiating strains involved in whooping cough outbreaks or even for characterization of standard laboratory strains.

Table II.1: Laboratory strains of Bordetellae genotyped by PFGE.

Strain	Type	Species	Source[a]
BP 338	A	*B. pertussis*	20
Maeno	A	*B. pertussis*	19
3779	B	*B. pertussis*	10
2753	C	*B. pertussis*	12
Sakairi	D	*B. pertussis*	19
134	b	*B. pertussis*	Wardlaw
11615	E	*B. pertussis*	ATCC
17903	na	*B. parapertussis*	10
110 H+	na	*B. bronchiseptica*	10

[a] Wardlaw, A. Wardlaw, Department of Microbiology, University of Galsgow, Glasgow, United Kindom; ATCC, American Type Culture Collection; na, type not assigned

FIGURE II.1

PFGE (CHEF) of *Xba* I digested laboratory strains of *B. pertussis* (lane 1 BP 338, lane 2 Maeno, lane 3 strain 3779, lane 4 strain 2753, lane 5 Sakairi, lane 6 MDH 134, lane 7 ATCC 11615), *B. parapertussis* (Bpp lane 8) and *B. bronchiseptica* (Bbs lane 9). Lanes represented by λ represent the λ ladder marker, a successively larger concatamer of 48.5 kb DNA fragments.

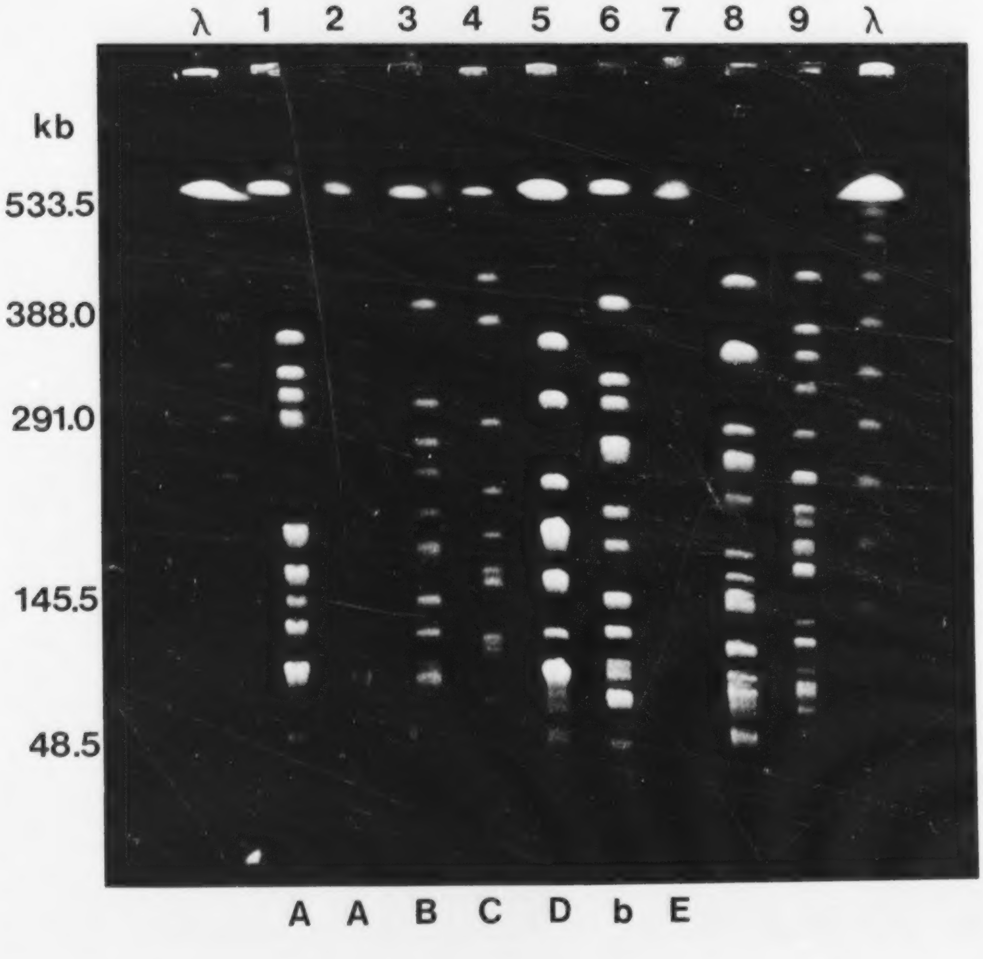

λ
1
2
3
4
5
6
7
8
9
λ
kb
533.5
388.0
291.0
145.5
48.5
A
A
B
C
D
b
E

FIGURE II.2

Clinical isolates digested by *Xba* I and resolved by PFGE. The isolates shown represent each of the 15 types generated by this study. Lanes 1 - 14 are clinical isolates each representing a unique DNA profile, or PFGE type, and corresponds with the letters shown on the map in Fig. 6. Lane 15 is the standard laboratory strain, *B. pertussis* 338. λ, molecular size marker (see Figure II.1 legend).

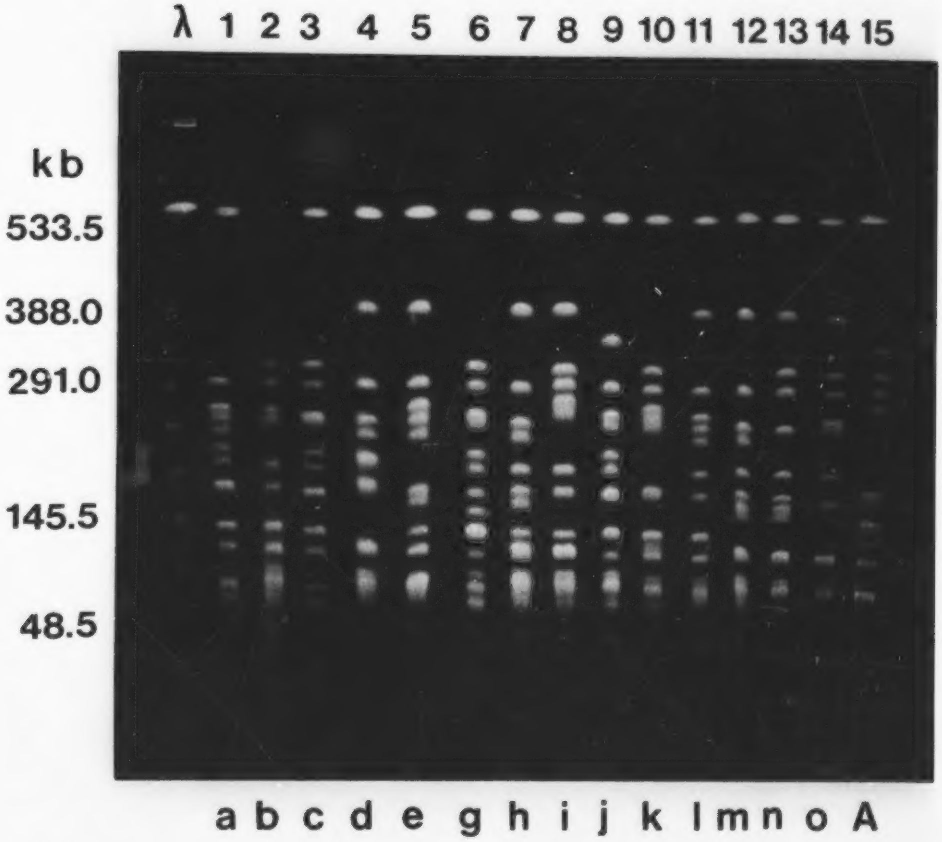

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kb
533.5
388.0
291.0
145.5
48.5
a b c d e g h i j k l m n o A

FIGURE II.3

Clinical isolates from siblings or parent-child pairs. Each pair have an identical PFGE type consistent with intra-familial transmission. Lanes 1 and 2, 7 year-old twins from Edmonton, Lanes 3 and 4, 3 year old and 8.5 year old brothers from Manning, Lanes 5 and 6, a 30 year old mother and her 13 year-old daughter from Edmonton, Lanes 7 and 8, a 2 year old boy and his 3 year old brother from Berwyn. Lanes 9 to 12 show two different pairs of siblings from High Level, 3 year old and 4 year old sisters and a 3 year old boy and his 1 year old sister, respectively. λ, molecular size marker (see Figure II.1 legend).

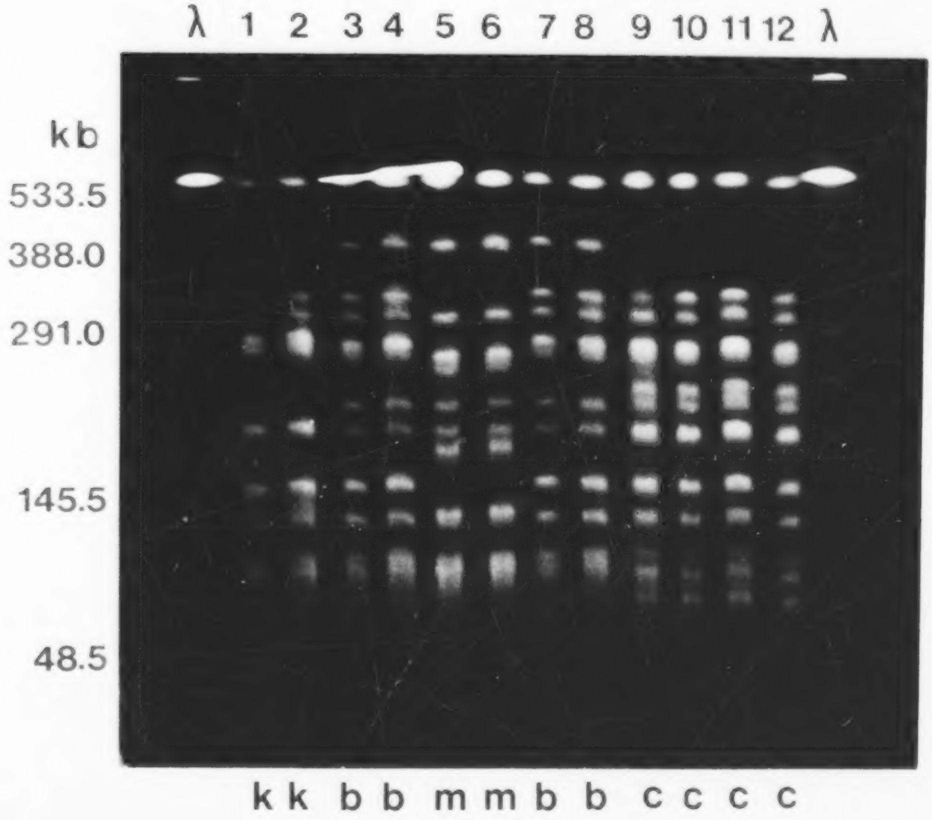

λ 1 2 3 4 5 6 7 8 9 10 11 12 λ
kb
533.5
388.0
291.0
145.5
48.5
k k b b m m b b c c c c

FIGURE II.4

Clinical isolates from Fort Smith, Alberta (number 1 on map in Fig. 6). Nine of 18 strains isolated within a three month period are shown, although all 18 possess the same PFGE type (data not shown). λ, molecular size marker (see Figure II.1 legend).

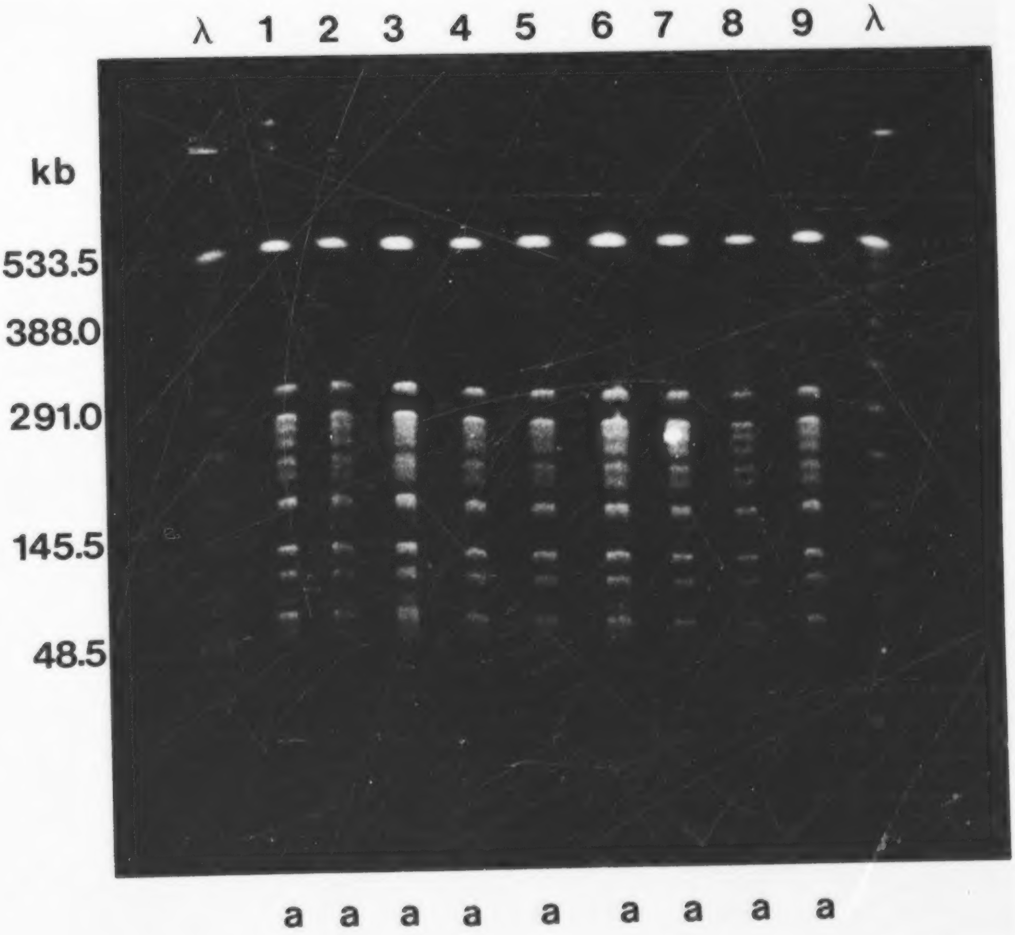

λ
1
2
3
4
5
6
7
8
9
λ
kb
533.5
388.0
291.0
145.5
48.5
a
a
a
a
a
a
a
a
a

FIGURE II.5

Clinical isolates from Whitehorse, The Yukon. Twelve strains isolate within a 12 month period are shown. Eleven of the twelve strains share the same PFGE type. λ, molecular size marker (see Figure II.1 legend).

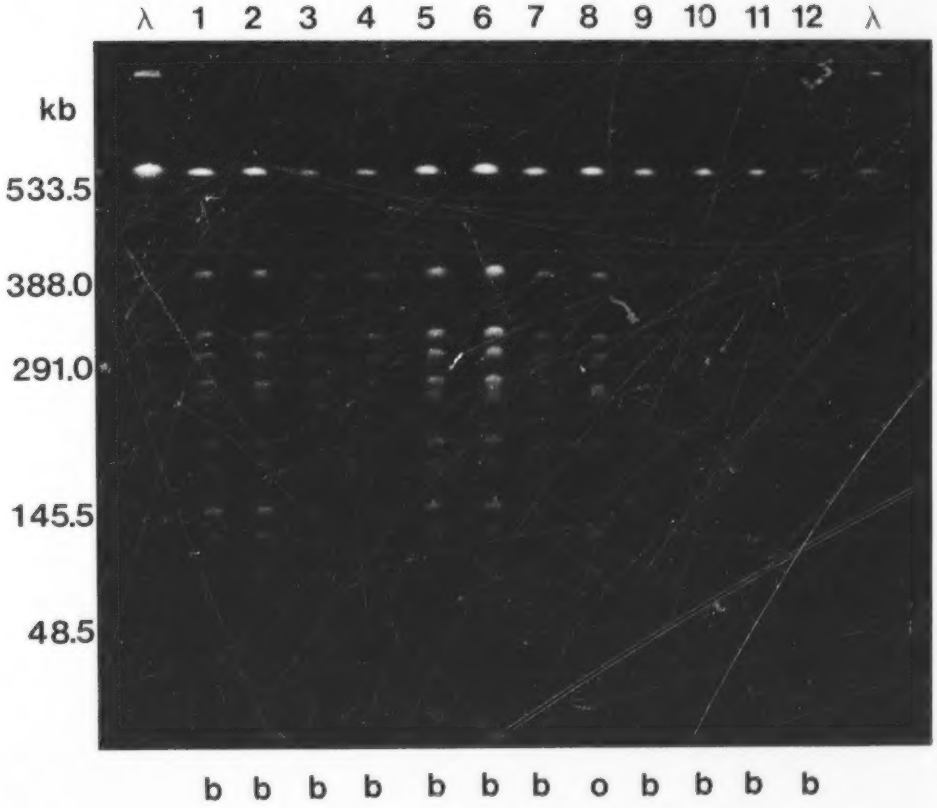
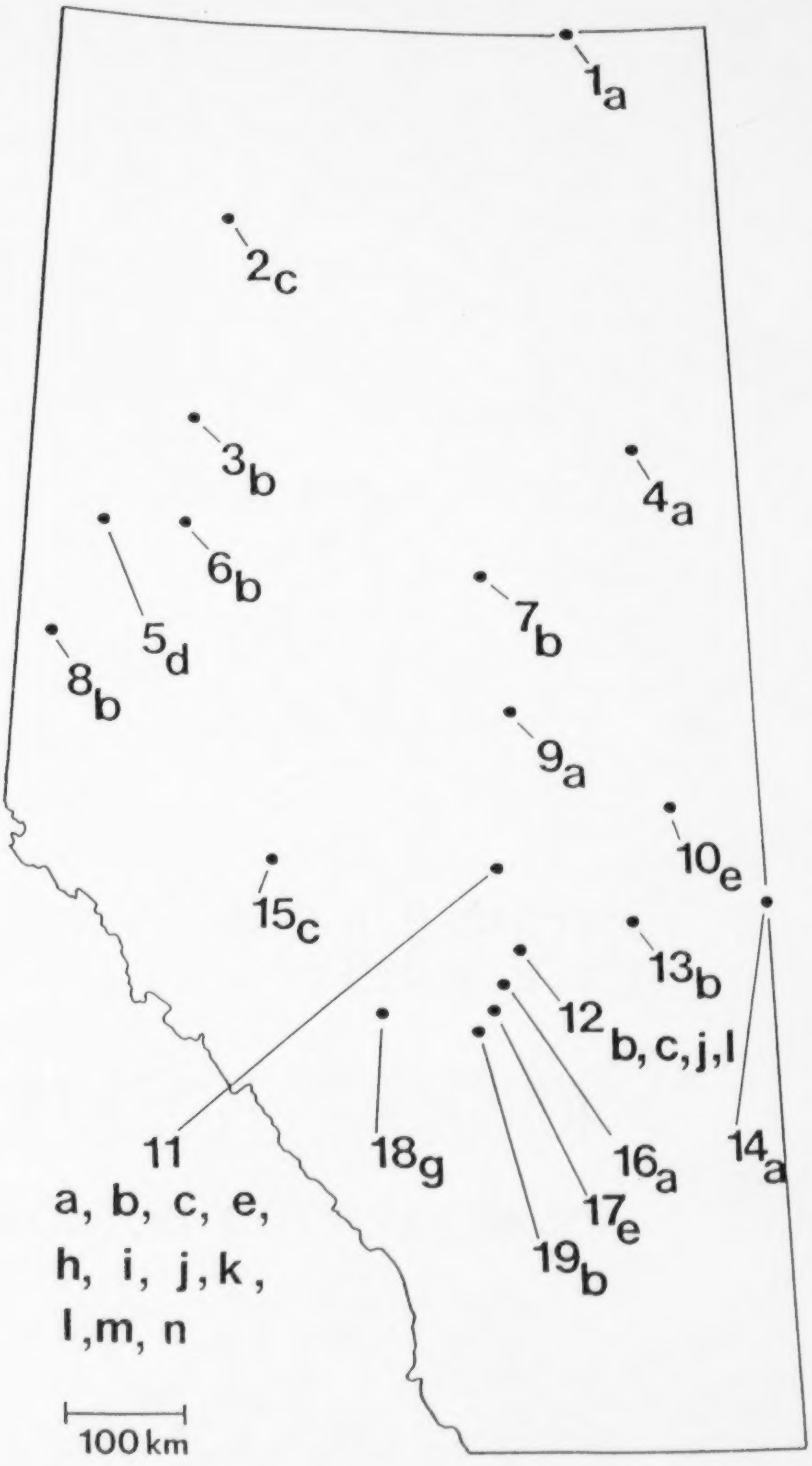

λ 1 2 3 4 5 6 7 8 9 10 11 12 λ
kb
533.5
388.0
291.0
145.5
48.5
b b b b b b b o b b b b

FIGURE II.6

Simplified geographic representation of the distribution of PFGE types identified across the province of Alberta (land area equivalent to 93% of that of the state of Texas). The numbers represent the following towns or cities: 1) Fort Smith 2) High Level 3) Manning 4) Fort McMurray 5) Hines Creek 6) Berwyn 7) Wabasca 8) Hythe 9) Athabasca 10) St. Paul 11) Edmonton 12) Hobbema 13) Viking 14) Lloydminster 15) Edson 16) Ponoka 17) Lacombe 18) Rocky Mountain House 19) Red Deer. The city of Edmonton's clinical isolates are shown to the lower left. Individual isolates from northern Alberta towns (eg: Edson, Hythe) were randomly chosen and may not necessarily reflect the prevalent PFGE types of their respective areas.



1a
2c
3b
4a
6b
5d
7b
8b
9a
10e
15c
13b
12 b, c, j, l
11
a, b, c, e,
h, i, j, k,
l, m, n
18g
16a
14a
17e
19b
100 km

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CHAPTER III

Effects of Different Enzymes and Resolution Parameters on PFGE Based Typing Systems.

A. INTRODUCTION

As shown in the previous chapter, pulsed-field gel electrophoresis (PFGE) has been applied to the epidemiological typing of *Bordetella pertussis*. Two different PFGE based typing systems exist for the epidemiological typing of pertussis. The first, was developed at the University of Manchester by Khattak and Matthews (2), and to date, at least 22 PFGE types, representing clinical isolates, exist (2, 4). The second system, our system, was developed at the University of Alberta (1), and to date, 20 PFGE types that represent clinical isolates exist. Both systems were developed at approximately the same time without knowledge by either group that PFGE was being applied to pertussis epidemiology. Each system uses the same restriction enzyme: *Xba* I, but is unique due to the different electrophoretic parameters used. An opportunity to compare both systems arose when 30 clinical isolates from Québec were analyzed first by Matthews' system, then our system. The results showed significant discordance between the systems.

In addition to the discordant results observed between these two systems, we have found further differences within our own system. When a sample of our two most predominant outbreak strains (type a and type b) were analysed further either by using other suitable enzymes, or by using higher resolution parameters of *Xba* I digests, strains that were once identical, no longer seem so. Furthermore, PFGE types designated as different by *Xba* I digestion of pertussis chromosomal DNA can be made to look identical by digesting DNA with *Spe* I or *Hpa* I. The emergence of these differences suggest the need to establish guidelines for rational PFGE DNA type designation. The use of defined enzymes and resolution parameters may have the limitation of suggesting identity between strains when in fact another enzyme may generate heterogeneity and demonstrate non-identity. Nevertheless, the use of standard PFGE guidelines allows the reproducibility of strain types between laboratories for the meaningful and sensitive analysis of epidemiological data where before now there was none.

Differences in PFGE patterns also pose questions concerning the relevance of PFGE based testing for *B. pertussis*. Are PFGE types a, b and c, predominant types because they possess pathogenic properties that are less expressed or absent in other types; or is their predominance simply the result of these strains existing as a large portion of the population of pertussis strains causing disease in Alberta? In a sense these questions also address the purpose of PFGE typing. Can this technique actually direct us to a population of potentially virulent pertussis strains; or is this simply a means of tracking the spread of the organism? Attempts at answering these questions have prompted the immunization of one group of mice with PFGE type a strain and another group of mice with PFGE type b strain. Western analysis of all 20 PFGE types with pooled, non-absorbed polyclonal anti-type a or anti-type b sera, revealed identical antibody binding profiles. These preliminary results suggest that the 20 different PFGE types of pertussis are antigenically identical.

B. MATERIALS AND METHODS

1. Bacterial strains and enzymes.

The clinical isolates were provided by the Provincial Laboratory of Northern Alberta, (Edmonton, Alberta, Canada). Québec isolates were provided by Manon Lorange, de Laboratoire de Santé Publique de Québec, (Québec, Canada). Organisms were cultured on Bordet-Gengou agar (Difco, Detroit, MI) using 15% defibrinated sheep's blood (Triage Labs, Ardrossan, AB) and incubated at 37°C in 98% humidity. Restriction enzymes used were: *Xba* I (Boehringer Mannheim Canada, Laval, Québec); *Spe* I and *Hpa* I (Sigma Chemical Co., St. Louis, MO).

2. Preparation, digestion, and resolution of agarose embedded DNA.

Chromosomal DNA was embedded in low melting point agarose to prepare DNA plugs for subsequent restriction endonuclease digestion and analysis by PFGE exactly as described in Chapter II of this thesis; the only difference was the CHEF apparatus used. For this study, we used a CHEF-DR™II system with a Pulsewave™ 760 Switcher powered by a model 200/2.0 constant voltage power supply (Bio-Rad

Laboratories, Missasauga, Ontario). For standard resolution, parameters included a 1% KILOrose gel (in 100 mL 0.5X TBE) with ramped pulse times from 5 to 45 seconds at a constant 175 V over a 24 hour run time at 14°C. These conditions consistently resolved DNA fragments in the range of 50 to 534 kb based on the DNA standard (Lambda Ladder PFG Marker, New England Biolabs, Missisauga, Ontario). For high resolution, parameters included a 1% KILOrose gel (in 100 mL 0.5X TBE) with ramped pulse times from 1 to 50 seconds at a constant 200 V over a 24 hour run time at 14°C. These conditions consistently resolved fragments in the range of 50 to 635 kb based on the DNA standard. The parameters described as "Matthews' parameters" consist of a 1% KILOrose gel (in 150 mL 0.5X TBE; 1X TBE: 10.3 g/L Tris base, 5.5 g/L boric acid, 0.93 g/L EDTA) with a static pulse time of 25 seconds at a constant 150 V for a run time of 40 hours at 14°C (2). These parameters consistently resolved fragments in the range of 50 kb to 415 kb based on the molecular weight marker. The gels were stained in 0.5 μg/mL ethidium bromide for 30 minutes and visualized by a UV transilluminator. The DNA bands were sized by comparison of migration distances to those of the DNA standard.

3. Production of non-absorbed, polyclonal mouse anti-type a and anti-type b sera (courtesy Lisa Purdy).

The recipient mice used for the immunization were BALB/c. Type a and type b strains of *B. pertussis* were cultured at 37°C on Bordet-Gengou agar (Triage Labs). Following culture, the organisms were killed by suspending them in a solution of 0.25% formalin in 1 X Tris glutamate saline (50 mM Tris-base, 50 mM Tris-HCl, 63 mM sodium glutamate, 43 mM NaCl, pH 7.5; TGS). The organisms were then pelleted and resuspended to an A_{540} of 0.200 in 0.1% merthiolate (Sigma Chemical Co., St. Louis, MO) in 1 X phosphate buffered saline (137 mM NaCl, 27 mM KCl, 80 mM monobasic sodium phosphate, 15 mM dibasic potassium phosphate; PBS). After a two week period of acclimatization, two groups of six mice were immunized. One group was immunized with type a whole organisms while the other was immunized with type b whole organisms. Each mouse was immunized intraperitoneally with 0.2 mL of bacterial suspension on days 0, 4, 8, and 12. On day 26, blood samples were removed by tail bleeds and antibody titres were determined to be greater than 1/10,000. On day 40, the mice were then boosted with the same innocula four more times with each immunization four days apart. Finally, on day 54, the antibody titres were reassessed and found to be quite high. The mice were sacrificed by cardiac puncture and the blood from each mouse was collected.

4. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western analysis of *B. pertussis* PFGE types with pooled mouse polyclonal antisera.

Duplicate suspensions of the twenty B. pertussis PFGE types were made in 1 X TGS to an A_{540} of 0.200, pelleted and resuspended in Laemmli's digestion mixture (5). The Laemmli suspensions were boiled for five minutes and loaded onto a Mini-Protean™ SDS-PAGE apparatus (Bio-Rad Laboratories, Missisauga, ON). The proteins were separated on a 16% polyacrylamide gel (30:0.1 acrylamide: bis-acrylamide) by running at constant power of 5 W over a one hour period after which the gels were removed and transferred electrophoretically (7) to nitrocellulose membranes (Bio-Rad). After transfer, the nitrocellulose membranes were removed and incubated overnight in 50 mL of blocking buffer (3% bovine serum albumin in 1 x PBS). The next day, 100 µL of pooled anti-type a or anti-type b was added to the blocking buffers (1/500 dilution) within which the appropriate membranes were incubated. The primary antibody was allowed to incubate for four hours at room tempertature with mixing. Excess primary antibody was removed by six ten minute washes with 1 x PBS. The secondary antibody used was a goat anti-mouse IgG with an alkaline phosphatase conjugate (Sigma) and 10 μL was added to 50 mL of fresh blocking buffer (1/5000 dilution). A four hour incubation at room temperature with mixing followed. Excess secondary antibody was removed by washing as previously described. The blots were developed by the addition of 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT).

C. RESULTS

1. Effects of different enzymes on strain designation.

By using our standard resolution parameters, we have observed that *Xba* I digests of type a and type b strains (Fig. III.1) produce homogeneous, type specific patterns. However, *Spe* I digests of the same type a and type b strains (Fig. III.2) causes 2 of 6 type a strains (lanes 2 and 4) to lack a 300 kb fragment found in the other four type a strains. Type b strains in the same figure showed a homogeneous pattern distinct from type a patterns. Thirdly, *Hpa* I digests of the same type a and type b strains as used in Fig. III.1, (Fig. III.3) produce homogeneous, type specific patterns.

Finally, *Spe* I and *Hpa* I digests were performed on two pairs of pertussis PFGE that differ by only one band with *Xba* I to see if these differences are consistent with the other enzymes. Types b and o differ from each other by one band when digested by *Xba* I (Fig III.4, lanes 1 and 2), as do types c and g (Fig. III.4, lanes 7 and 8). However when *Spe* I and *Hpa* I digests of these types are resolved (Fig. III.4), types b and o remain as unique types (lanes 3 - 6), whereas types c and g seem identical when digested by *Spe* I (lanes 9 - 10) but different when digested with *Hpa* I (lanes 11-12).

2. Effects of high resolution parameters on strain designation.

When high resolution PFGE parameters were applied to *Xba* I digests (Fig. III.5), we observed that a large fragment, once assumed to be a single fragment due to the map of *B. pertussis* Tohama I (6, Fig. 4), can be resolved into one to three fragments, none of which are conserved nor identical between type a and type b strains.

3. Comparison of the UA PFGE system to the Matthews' PFGE system.

For convenience, we refer to our standard parameters as the UA PFGE system. We compared the UA PFGE to the Matthews' PFGE system by analysing 30 clinical isolates from Québec. The isolates were first typed by Matthews' system (not shown), then sent to us blind to be typed by our system (Figures III.6a and III.6b). Analysis of the Québec isolates by the UA system revealed that 18 of the 30 strains fell into five types already established within the UA system (types a, b, c, j, and l). The frequency of these five strains were different than anticipated with types c and l predominating, comprising 8 (27%) and 5 (17%) of the 30 strains respectively. Types a, b, and j comprised 2, 2, and 1 of the 30 strains respectively. The remaining 12 Québec strains consisted of six new UA PFGE types which we assigned p, q, r, s, t, and u with a frequency of 4, 4, 1, 1, 1, and 1 respectively.

After we had sent our results to Montreal, we obtained the results observed by Ruth Matthews and sent to Kathleen

Knowles (4). Table III.1 compares the results of PFGE typing 30 Québec strains by the UA (UA) and the Matthews' (M) systems. There is considerable discordance between the two systems. For example, of the four UA-type p strains, two are M-type 22, one is M-type 5, and one is M-type 'unique'. These findings are further complicated because all four of the UA-type q strains are assigned as M-type 22, and a UA type 1 strain as well as six of the eight UA-type c strains are designated as M-type 5. The data obtained by the Matthews' system is not truely informative due to the assignment of 'unique' to a PFGE type not previously determined by their system. Thus, we cannot be certain if, for example, the two UA-type a strains from Québec are the same as each other or not because they were both designated as M-type 'unique'. In an attempt to visualize the uniqueness of M-type strains, we used the running parameters and buffer of Matthews with our apparatus. Of the 30 Québec strains, 19 strains, consisting mainly of 'unique' designation, were analysed in this manner (Fig. III.7). We compared the patterns of strains Q12670 (UA-type 1, M-type 1) and Q12649 (UA-type c, M-type 5) to the type 1 and 5 patterns in Figure 1 of the paper by Khattak et al. (). This confirmed that we reproduced their parameters faithfully. The analysis of the 19 Québec strains under

Matthews' parameters, was not able to resolve the discordance between the two systems. Firstly, UA-type a and UA-type b strains when run with Matthews' parameters produced homogeneous patterns distinct from each other (Fig. III.7, lanes 1 - 4). The two UA-type c strains, one M-type 5 and the other M-type 'unique', are identical (Fig. III.7, lanes 5 and 6). The four UA-type l strains, two M-type 'unique' and the other two M-type 1 and 5, are all identical (Fig. III.7, lanes 7 - 10). Fourthly, the three UA-type p strains: one M-type 5, one M-type 22 and the other M-type 'unique', are all identical but distinct from UA-type q which was M-type 22 (Fig III.7, lanes 11 - 15). Finally, UA-types r, s, t, and u (all M-type 'unique') produced distinct patterns with Matthews' parameters (Fig. III.7, lanes 16 - 19).

4. Serological strain comparison using mouse polyclonal antibodies.

The sera collected from cardiac puncture of the sacrificed mice were pooled so that there was type a pooled antiserum and type b pooled antiserum. The protein profiles of the 20 pertussis PFGE types were resolved by SDS-PAGE and transferred to nitrocelluose for analysis with Western blot using the pooled mouse antisera as the primary antibody. The results of this analysis was that each of the 20 PFGE types showed no differences with either antibody preparation (Fig III.8).

D. DISCUSSION

Due to the lack of reliable typing systems for Bordetella pertussis (e.g.: serology, multilocus enzyme electrophoresis, ribotyping), a PFGE-based typing system has been shown to be a promising alternative. Using the UA standard parameters on *Xba* I digests of two reference laboratory strains of pertussis (Tohama I and Maeno), an identical pattern was generated for these two strains. Upon digestion with *Hpa* I or *Spe* I, these two strains showed conserved, identical patterns that were distinct respective to the other two enzymes. With these results, we felt confident that strains, typed as identical by one enzyme, namely *Xba* I, should remain so when subject to analysis by other enzymes such as *Hpa* I and *Spe* I. As shown here, this is not the case when *Spe* I digests of six UA-type a strains were subjected to analysis by standard PFGE conditions (Fig. III.2) or when types c and g are analysed by *Spe* I or *Hpa* I

(Fig III.4, lanes 9 - 12). In addition, high resolution parameters used to resolve *Xba* I digests have separated a presumed single large fragment into one to three fragments, potentially giving rise to three subtypes of a and b strains.

The emergence of these minor discrepancies either with the use of different enzymes or different parameters may potentially create confusion within an already extremely sensitive system for *B. pertussis* strain differentiation. Since the discrepancies described within our system are relatively minor, we believe that our typing system can produce reliable data using standard parameters to resolve *Xba* I digests of *B. pertussis* chromosomal DNA.

However, as shown by Table III.1, the discordance observed between the UA and Matthews' systems is not minor. There are clear and obvious differences between the four UA-type p strains and the four UA-type q strains (Fig III.6b, Fig III.7), warranting their classification into two distinct UA types. These differences are not observed by the Matthews' system. All four of the type q strains and two of the type p strains are M-type 22, and the other two type p strains are M-type 'unique' and M-type 5. Also, the obvious similarity within the 8 UA-type c strains (Fig.

III.6a, Fig. III.7) and within the 5 UA- type 1 strains (Fig. III.6b, Fig. III.7), is not demonstrated by the Matthews' system. As shown by Figure III.7, we have not been able to resolve these discrepancies. There may be two explanations for the disagreement: 1) the 30 strains received from Québec were labelled incorrectly; 2) the assignment of molecular weights to fragments in the Matthews' system was incorrect. According to table 1 in the paper by Khattak et al. (2), DNA type by the Matthews' system has any of the following bands (in kb): 412, 375, 340, 315, 280, 271, 256, 240, 224, 215, and 200. For example, type 1 has the following seven bands (in kb): 412, 315, 280, 271, 256, 224, and 200 (2, Table 1). However, Type 7 has seven bands (in kb): 412, 340, 315* (*band runs at 297), 280, 271⇑ (⇑band runs 275), 224 and 220 (2, Table 1). This obscure and arbitrary use of symbols occurs with other types as well (2, 3) and may a reason for the differences between the two systems. Due to the lack of reference strains within the Québec isolates (i.e.: types 1 and 5, symbols not assigned), the exploration of this possibility has not been attempted. Unfortunately, an exchange of strains to rectify these differences has yet to be arranged.

The data obtained by the Western analysis of the twenty pertussis PFGE types addresses the relevance of PFGE as an epidemiological tool. Preliminary results (courtesy Lisa Purdy) consisted of Western analysis of UA-type a and b strains by antiserum from each individual mouse. Each profile was different, but it was later determined that this was due to the variation of the response by each mouse. For example, a mouse immunized with PFGE type a organisms produced polyclonal sera that recognized the same epitopes on electrophoretically resolved type a and type b antigens. When the other mice from the same group were immunized with the same type a organisms, each mouse produced polyclonal sera that recognized different epitopes from the same electrophoretically resolved type a and type b antigens but still could not distinguish between the type a and type b strains. Upon pooling the sera from each group, we observed that there was no variation between strains. The antisera used was non-absorbed, that is, antibodies common to all strains were most likely present, thus masking any variable antibodies if present. A technique such as immunoelectrophoresis could be modified such that the antigens of a PFGE type b strain are separated electrophoretically in one dimension before absorbing the common antibody clones of anti-type a sera in the second dimension. After absorption, any unique antibody clones (now specific for antigens unique to type b strains) remaining in the intermediate gel are electrophoresed into an adjacent gel and allowed to interact with type b organisms contained within the gel.

This chapter has shown that PFGE typing of *B. pertussis* is prone to intra and inter system discordance. By creating a standardized set of parameters steps can be taken to eliminate future confusion in pertussis genotyping by this method.

Table III.1: Type comparison of Québec isolates using UA and Matthews' PFGE systems.

Strain	**UA Type**	**Matthews' Type** (4)
Q12776	**a**	**'unique'***
Q12787	**a**	**'unique'**
Q12673	**b**	**'unique'**
Q12766	**b**	**'unique'**
Q12649	**c**	**5**
Q12683	**c**	**'unique'**
Q12701	c	'unique'
Q12770	c	5
Q12789	c	5
Q12809	c	5
Q12817	c	5
Q12820	c	5
Q12783	**j**	**'unique'**
Q12650	**l**	**'unique'**
Q12670	**l**	**1**
Q12677	**l**	**'unique'**
Q12686	l	1
Q12695	**l**	**5**
Q12640	**p**	**22**
Q12644	**p**	**'unique'**
Q12645	**p**	**5**
Q12802	p	22
Q12706	**q**	**22**
Q12772	q	22
Q12806	q	22
Q12814	q	22
Q12659	**r**	**'unique'**
Q12702	**s**	**'unique'**
Q12761	**t**	**'unique'**
Q12763	**u**	**'unique'**

* 'unique' refers to a strain not seen before by the Matthews' system.

- boldface type indicates those strains analysed with Matthews' parameters on Fig. III.7

FIGURE III.1

Xba I digests of type a and type b strains resolved by standard PFGE parameters. Six type a and eight type b strains were digested by *Xba* I and resolved by PFGE using standard parameters as described in materials and methods. **λ**, lambda ladder marker, a successively larger concatamer of 48.5 kb DNA fragments.

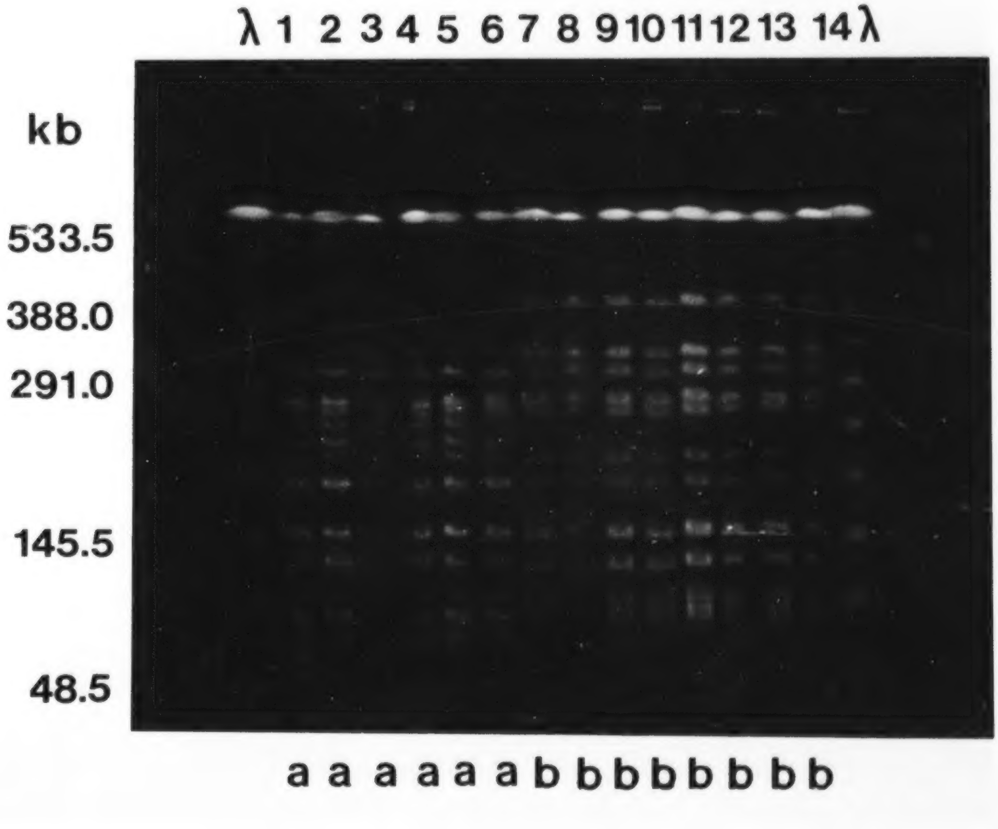

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 λ
kb
533.5
388.0
291.0
145.5
48.5
a a a a a a b b b b b b b b

FIGURE III.2

Spe I digests of type a and type b strains resolved by standard PFGE parameters. The same six type a and eight type b strains were digested by *Spe* I and resolved by PFGE using standard parameters described in materials and methods. Lanes 2 and 4 show type a strains lacking a 300 kb fragment present in the other four strains. The white area in the 300 kb region of lane 5 is excess ethidium bromide which masks the two bands of that region. Type b strains are all identical. λ, lambda ladder, see Fig. III.1 legend.

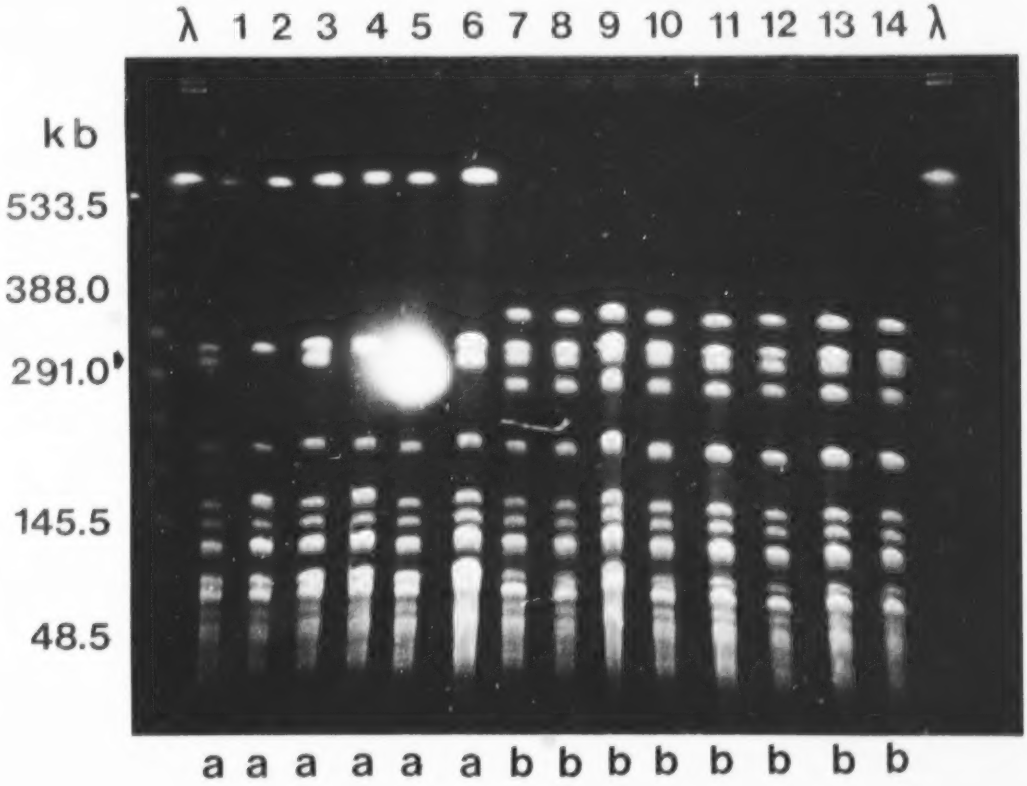

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 λ
kb
533.5
388.0
291.0
145.5
48.5
a a a a a a b b b b b b b b

FIGURE III.3

Hpa I digests of type a and type b strains resolved by standard PFGE parameters. The same six type a and eight type b strains were also digested with *Hpa* I and resolved by PFGE using standard parameters described in materials and methods. All six type a and eight type b strains produced homogeneous, yet type specific patterns. λ, lambda ladder, see Fig. III.1 legend.

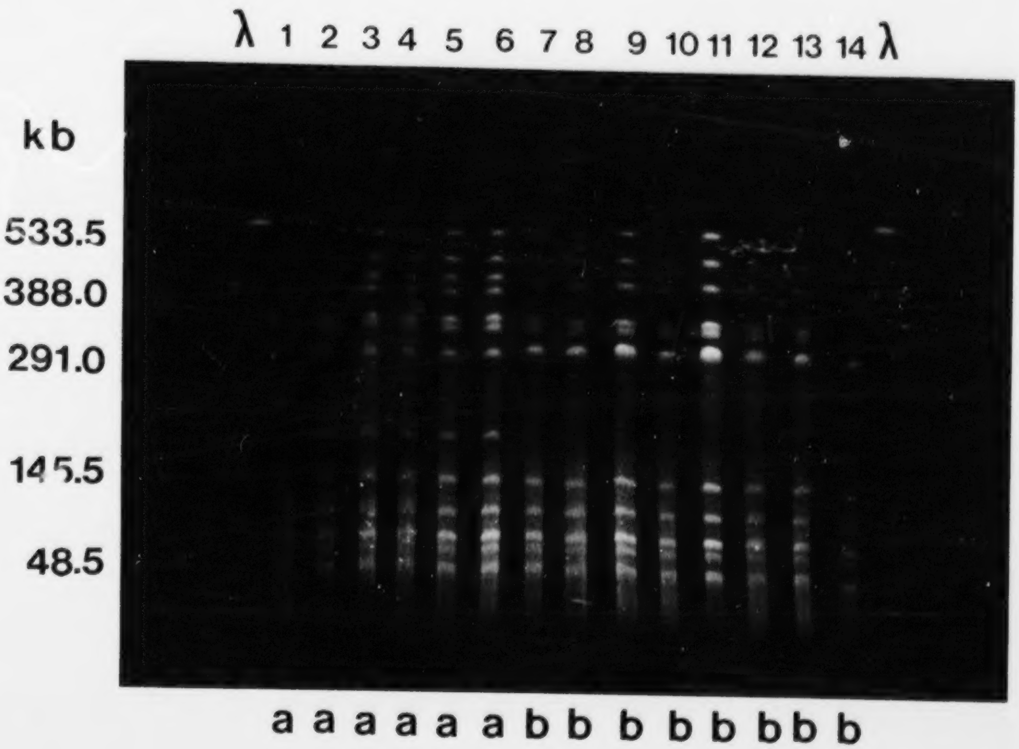

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 λ
kb
533.5
388.0
291.0
145.5
48.5
a a a a a a b b b b b b b b

FIGURE III.4

Xba I (X) types b, o and c, g digested with *Spe* I (S) and *Hpa* I (H). *Xba* I types differing by one band: b, o (lanes 1 and 2) and c, g (lanes 7 and 8) were separately digested by *Spe* I and *Hpa* I and resolved by PFGE using standard parameters as described in materials and methods. Types b and o remain as unique types when digested by *Spe* I (lane 3 is sheared, therefore comparison was made by refering to Fig. III.2.) and *Hpa* I. Types c and g however, look identical when digested with *Spe* I (lanes 9 and 10) but different when digested by *Hpa* I (lanes 11 and 12). λ, lambda ladder, see Fig. III.1 legend.

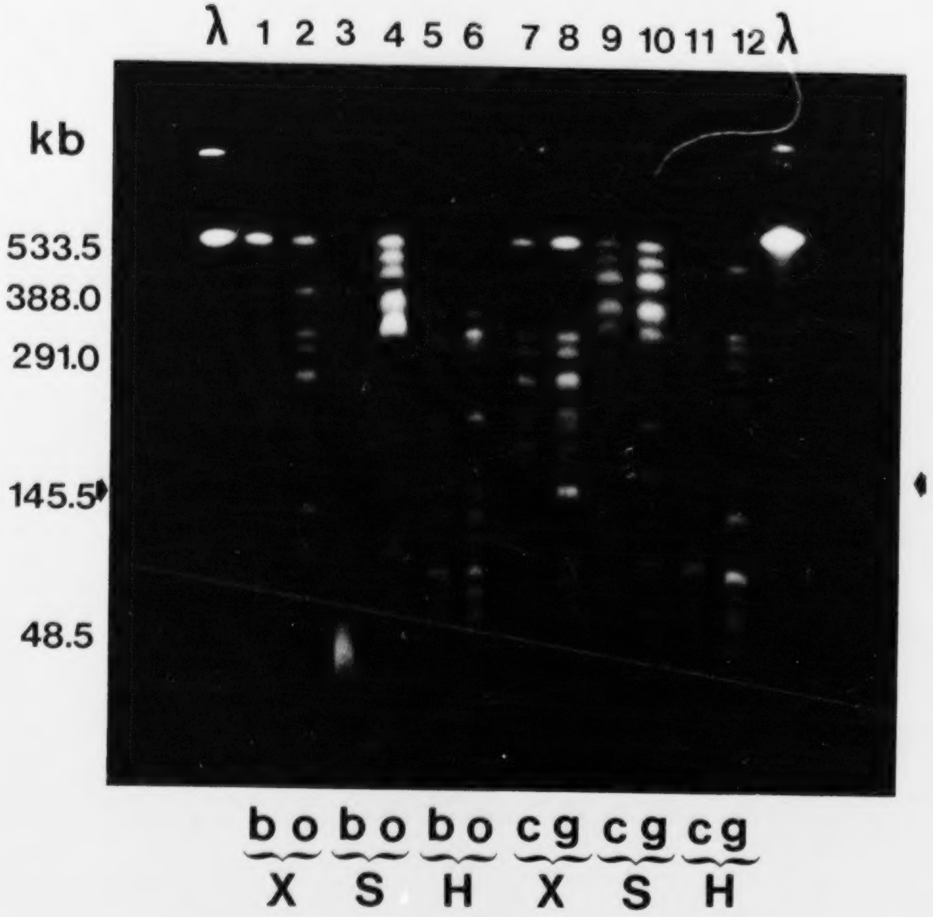

λ 1 2 3 4 5 6 7 8 9 10 11 12 λ
kb
533.5
388.0
291.0
145.5
48.5
b o b o b o c g c g c g
X S H X S H

FIGURE III.5

Xba I digests of type a and type b strains resolved by PFGE using high resolution parameters. The same six type a and eight type b strains as in Figures III.1-3 were digested with *Xba* I and resolved by PFGE using high resolution parameters as described in materials and methods. A once assumed large fragment at about 540 kb has been resolved into one (as shown by lane 1), two (as shown by lanes 2 - 5, 7 - 11, 13 and 14), or three (as shown by lanes 6 and 12) fragments. λ, lambda ladder, see Fig. III.1 legend.

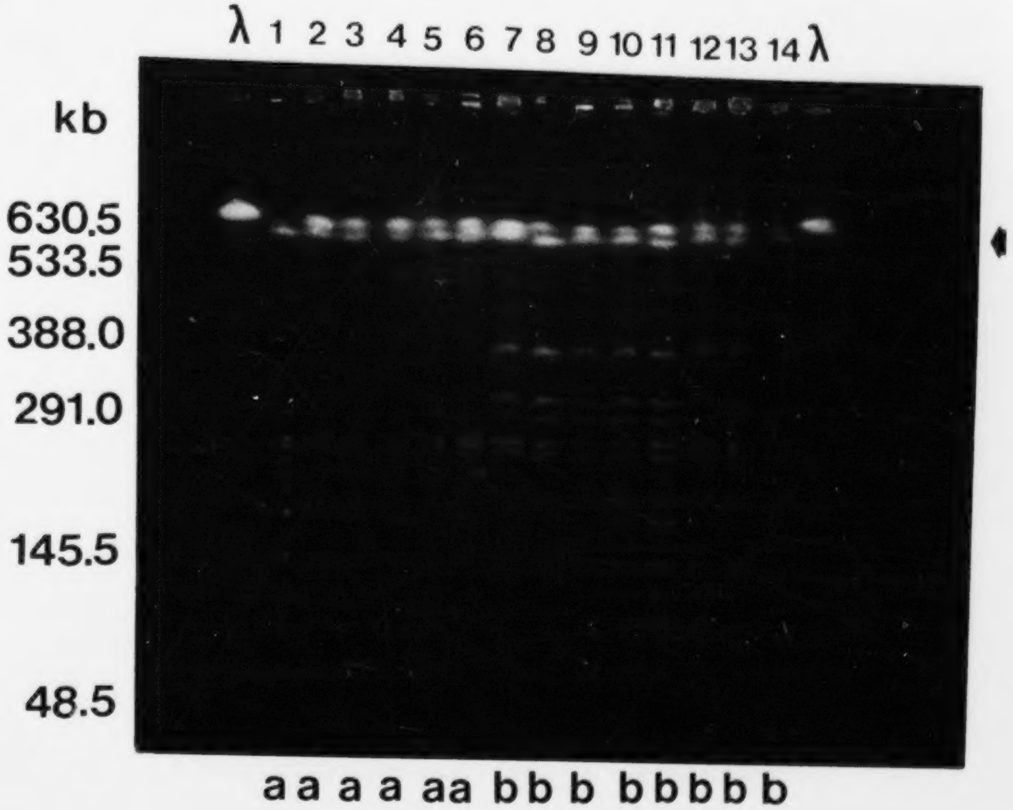

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 λ
kb
630.5
533.5
388.0
291.0
145.5
48.5
a a a a a a b b b b b b b b

FIGURE III.6a

Xba I digests of Québec isolates. Québec isolates were digested with *Xba* I and resolved by PFGE using standard parameters as described in materials and methods. UA types a, b, c, and j (lanes 1, 4, 7, and 16, respectively), were included as reference types to confirm identity. λ, lambda ladder, see Fig. III.1 legend.

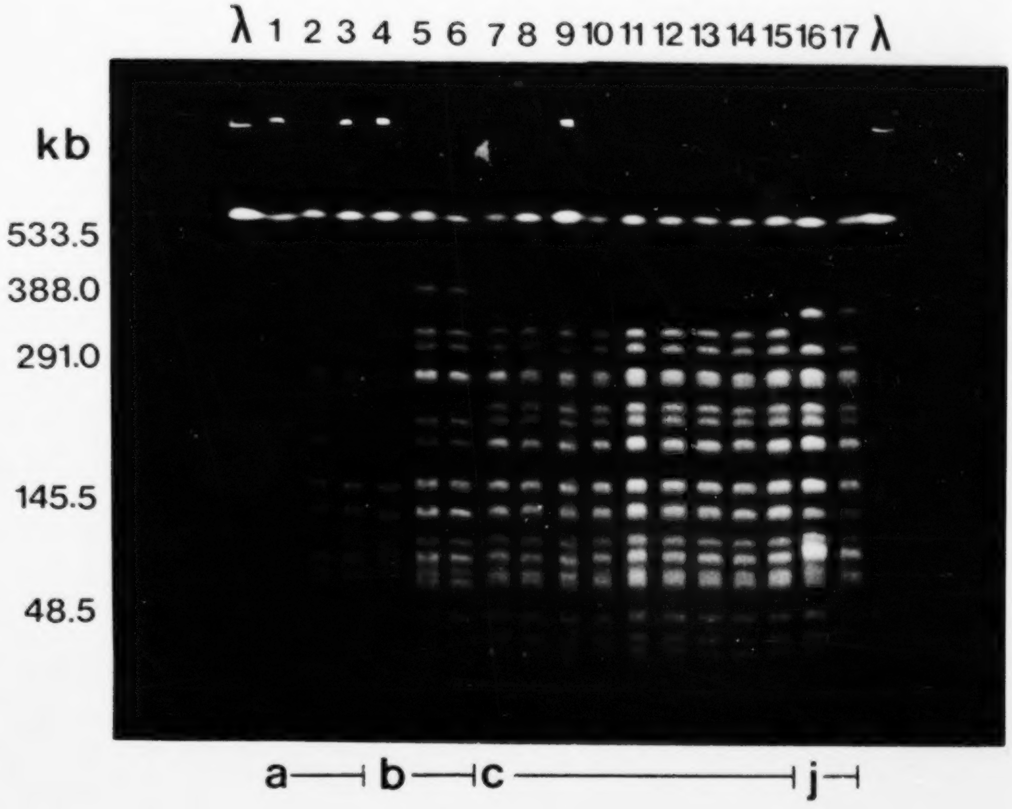

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 λ
kb
533.5
388.0
291.0
145.5
48.5
a
b
c
j

FIGURE III.6b

Xba I digests of Québec isolates. Québec isolates were digested with *Xba* I and resolved by PFGE using standard parameters as described by materials and methods. UA type 1 (lane 1) was included as a reference to confirm identity. Types p, q, r, s, t, and u are types new to the UA system.

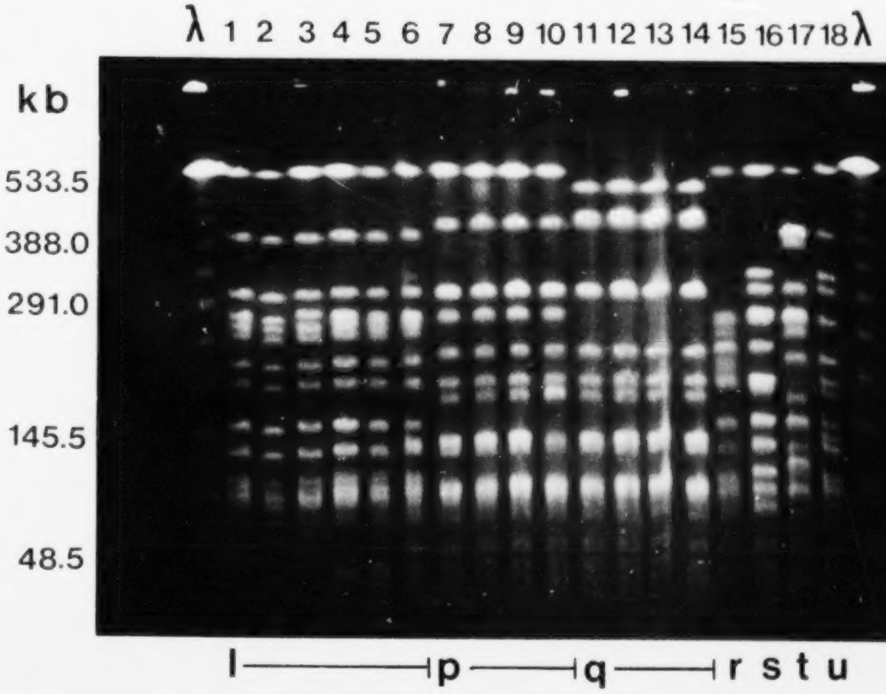

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 λ
kb
533.5
388.0
291.0
145.5
48.5
l
p
q
r s t u

FIGURE III.7

Analysis of 'unique' strains by PFGE. Québec strains, typed as 'unique' by Matthews' (M) system were digested by *Xba* I and resolved by PFGE using Matthews' parameters as described in materials and methods. The black dot represents all strains designated 'unique' by Matthews' parameters (see Table III.1). The UA designations are based on patterns identified by the work done in this thesis.

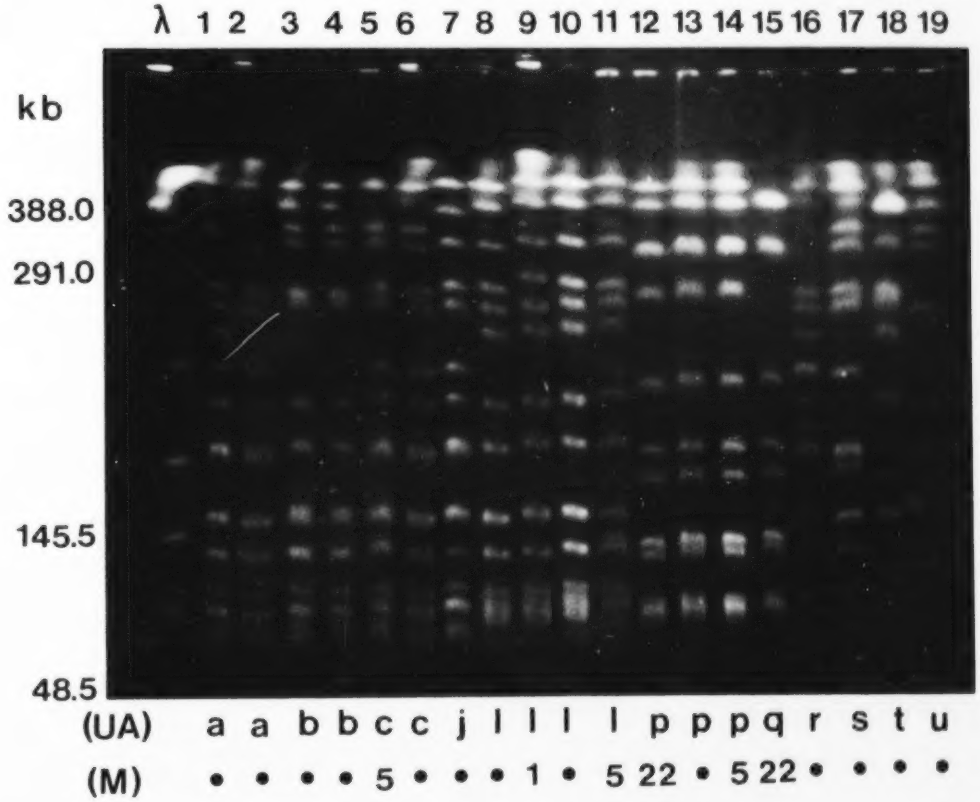
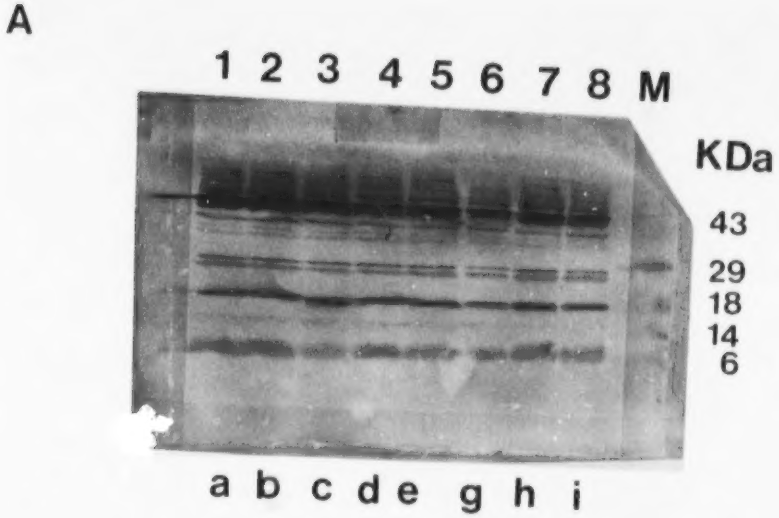
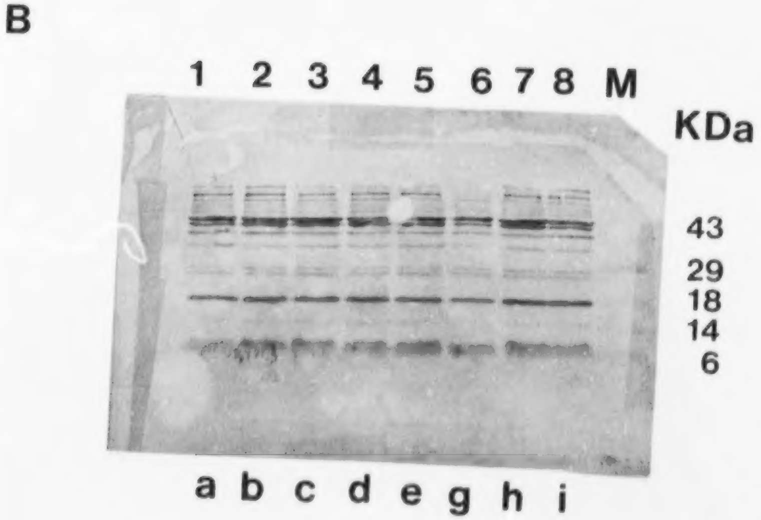

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kb
388.0
291.0
145.5
48.5
(UA) a a b b c c j l l l l p p p q r s t u
(M) • • • • 5 • • • 1 • 5 22 • 5 22 • • • •

FIGURE III.8

Western analysis of *B. pertussis* PFGE types a through h using: A) non-absorbed polyclonal anti-type a pooled sera and B) non-absorbed polyclonal anti-type b pooled sera. All twenty PFGE types showed no differences with either antibody (data not shown).


A
1 2 3 4 5 6 7 8 M
KDa
43
29
18
14
6
a b c d e g h i


B
1 2 3 4 5 6 7 8 M
KDa
43
29
18
14
6
a b c d e g h i

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CHAPTER IV

Random-Amplified Polymorphic DNA (RAPD) Fingerprinting of *Bordetella pertussis*.

A. INTRODUCTION

The ability to track clinical isolates of *B. pertussis* using pulsed-field gel electrophoresis has been an important development in the epidemiology of whooping cough. However, a major pitfall to PFGE typing is that it is labor intensive and time consuming. Twenty pertussis isolates typed by PFGE takes approximately five to six days. This method is also expensive: each strain analysed costs $15.00 which does not include the cost of purchasing a PFGE apparatus ($12,000.00 to $15,000.00). Considering that there are well over 500 clinical isolates remaining to be analysed from the 1991 whooping cough outbreak alone, the implementation of a fast, inexpensive and reasonably discriminatory (as PFGE) method for pertussis epidemiology is an important priority.

The use of the polymerase chain reaction for bacterial epidemiology has become an attractive alternative for many genera. Primers designed to amplify chromosomal DNA may be specific as in the case of IS6110 of *Mycobacterium* *tuberculosis* (9), or random, as in the case of *Helicobacter pylori* (1). Due to either the conserved nature of *B. pertussis* insertion sequences (3) or to the infrequency of variable ones (8), the use of insertion sequence specific primers is insensitive for strain discrimination. However, the success with random-amplified polymorphic DNA's (RAPD's) with other bacterial genera such as *H. pylori* (1), *Candida albicans* (2), *Yersinia pseudotuberculosis* (6), and *Pseudomonas aeruginosa* (5), has prompted the exploration of this method as a potential typing method for *B. pertussis*. Compared to PFGE for pertussis typing, RAPD's are faster because the twenty samples that took six days to analyse by PFGE could be analysed within 48 hours by the RAPD technique. Also, the cost per RAPD assay ($3.00) is about five times less that of PFGE assay ($15.00)

The principle behind the RAPD PCR technique is based upon lowering the annealing temperature (36°C compared to 60°C of the regular PCR cycle) to allow short, non-specific ten to twelve base pair oligonucleotides to randomly anneal to sheared chromosomal DNA. Random annealing for a given bacterial strain is very reproducible (1, 3, 5) from assay to assay. A minor pitfall of RAPD's however, is the lack of reproducibility when different thermocyclers and/or Taq polymerases are used (5, 7). For example, when using the Perkin Elmer 480, reproducible patterns for *Pseudomonas* strains are generated. When using the Perkin Elmer 6000, however, reproducible patterns for *Pseudomonas* are also generated but are different from those created by the Perkin Elmer 480 (5).

The aim of this chapter is to demonstrate the feasibility of the RAPD technique for pertussis epidemiology and to compare its strain discriminating power to the established PFGE technology. Primers that have generated complex, strain specific patterns for organisms such as *Pseudomonas* and *Mycobacterium* were tested with pertussis PFGE type a, b, and c strains. While the primers generated complex patterns, the patterns were essentially identical for the three strains that were screened. One primer was used on nine different PFGE types (a through j) and all nine gave identical RAPD patterns. This preliminary data suggests that RAPD analysis of *B. pertussis* may not be informative for the epidemiology of whooping cough.

B. MATERIALS AND METHODS

1. Bacterial strains, enzymes, and oligonucleotides.

The clinical isolates were provided by the Provincial Laboratory of Northern Alberta, (Edmonton, Alberta, Canada). Québec isolates were provided by Manon Lorange, de Laboratoire de Santé Publique de Québec, (Québec, Canada). Organisms were cultured on Bordet-Gengou agar (Difco, Detroit, MI) using 15% defibrinated sheep's blood (Triage Labs, Ardrossan, AB) and incubated at 37°C in 98% humidity. Laboratory strain *Pseudomonas aeruginosa* PAK was obtained from Dr. Randy Irvin (department of MMID). Taq polymerase was obtained from Gibco BRL (Burlington, ON). Primers for RAPD analysis were purchased as set #3 consisting of 100 random oligonucleotides (10-mers; #201-#300) was obtained from the University of British Columbia Oligonucleotide Synthesis Laboratory (Vancouver, BC).

2. Preparation of sheared chromosomal DNA.

The isolation of sheared DNA was achieved as described by Mahenthiralingam et al. (5). Bacterial suspensions were made of pertussis PFGE types a through u and *P. aeruginosa* PAK by suspending each strain to an A_{540} of 0.600 in 1 X TE

(10 mM Tris-base, pH 8.0, 1 mM EDTA). Bacteria from 1.5 mL of each suspension was harvested by centifugation for 5 minutes at 10,000 rpm. The pellets were resuspended in 0.4 mL of GET buffer (50 mM glucose, 70 mM EDTA, 50 mM Tris-HCl pH 8.0). Of this suspension, 0.2 mL was added to 0.9 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS, 30 µg/mL RNAse) in a 2.0 mL Eppendorf tube containing approximately 0.5 mL of sterile, acid washed 0.1 mm glass beads (Sigma Chemical Co., St. Louis, MO). The mixture was vortexed for 1 minute and the lysate incubated for 1 hour at 37°C. After the incubation, the tubes were centrifuged for 2 minutes at 8,000 x g. Protein and polysaccharide contaminants were precipitated from 0.7 mL of the cleared lysate by the addition of 0.235 mL of saturated ammonium acetate followed by thorough mixing and centrifugation at 8,000 x g for 5 minutes at room temperature. The DNA was precipitated from 0.4 mL of the supernatant by adding 0.2 mL of ice cold (-20° C) isopropanol followed by centifugation at 8,000 x g for 5 minutes. DNA concentration was estimated by $A_{260/280}$ and the sample was diluted to 20 ng/µL in 1 X TE in preparation for PCR.

3. RAPD analysis.

The polymerase chain reactions were set up as described previously (1), with the only modification being the use of the manufacturer's PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl) instead of 10 mM Tris-HCl pH 8.0, 50 mM KCl. Each reaction contained the following in a final volume of 25 µL: 40 ng of either *B. pertussis* or *P. aeruginosa* DNA, 40 pmol of oligonucleotide, 3.5 units of Taq polymerase, 250 µM of each deoxynucleoside triphosphate (Boehringer Mannheim, Laval, Québec), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.001% gelatin and 3 mM $MgCl_2$ (overlaid with 25 µL of sterile, light mineral oil). The reactions were amplified using a Techne thermocycler as follows: a) 4 cycles at 94°C, 5 min.; 36°C, 5 min.; 72°C, 5 min; b) 30 cycles at 94°C, 1 min.; 36°C, 1 min.; 72°C, 2 min.; followed by a final extension at 72°C for 10 minutes. A 10 µL fraction of each reaction was then analysed by standard agarose gel electrophoresis in 0.7% agarose and 0.4% Synergel (Diversified Biotech, Newton Centre, MA) in 0.5 X TBE (0.9 M Tris-borate, 0.02 M EDTA; 5 X TBE). PCR products were sized by comparison to a standard curve based upon the mobility the molecular weight markers (1 kb ladder, Gibco BRL, Burlington, ON).

C. RESULTS

1. RAPD analysis of pertussis PFGE types.

PFGE types a, b, c, and *P. aeruginosa* PAK were analysed with the RAPD technique using primers 208, 228, 241 (data not shown), 270, 272, 275, 277, 284, and 287 (Figs. IV.1, IV.2, and IV.3). The patterns generated from each PFGE type by each of the primers are complex, but overall, they are not distinct from each other. In the case of primer 228, there is a one band difference between PFGE type a and b, however, PFGE type c is identical to PFGE type b (Fig. IV. 1, lanes 5 -7). Primer 275 shows a one band difference between PFGE types a and b also, however PFGE type c remains identical to PFGE type b (Fig. IV.1, lanes 13 - 15).

The question as to the reproducibility of these variable bands has been addressed thanks to the work of Dr. Eshwar Mahenthiralingam. With his permission, the data showing RAPD analysis of pertussis PFGE types a through j with primer 272 is figure IV.3 of this thesis. This data shows that with primer 272, the different pertussis PFGE types are identical with the exception of one band at approximately 1.0 kb. This band is most likely non-specific due to its presence in only one of the duplicate j strains that were assayed (Fig. IV.3, lanes 9 and 10).

P. aeruginosa PAK was included with each of the primer screens as a positive control. Direct comparisons between our PAK patterns and those of Dr. Mahenthiralingam could not have been made due to the different thermocyclers used.

D. DISCUSSION

The use of a PCR-based technique for pertussis epidemiology as an alternative to PFGE was explored in this chapter. Random-amplified polymorphic DNA PCR is a fast and inexpensive alternative and has been successful with other organisms (1, 2, 5, 6). Its only drawback is the lack of inter-thermocycler and inter-polymerase reproducibility (6).

We have shown here that after preliminary analysis, the RAPD technique is, pending further research, unsuitable for fingerprinting pertussis DNA. The primers used for this study have shown clear and obvious differences with organisms such as *Pseudomonas* and *Mycobacterium* (5), but were unable to show differences within the three predominant pertussis PFGE types. Due to time constraints, other primers could not be screened.

Our results seem to be consistent with the existing findings of the lack of variability within the organism *Bordetella pertussis*. A consistent theme has been established throughout this thesis in that, of the many molecular typing methods available, virtually all of them are able to show differences in bacterial strains from many genera except *Bordetella pertussis*. It is hardly surprising that the preliminary data shown with the RAPD technique does not show any differences between pertussis strains.

FIGURE IV.1

RAPD primer screen of primers 208, 228, 270, and 275 on pertussis PFGE types a, b, and c and *P. aeruginosa* PAK (labelled K). M: 1 kb molecular weight marker.

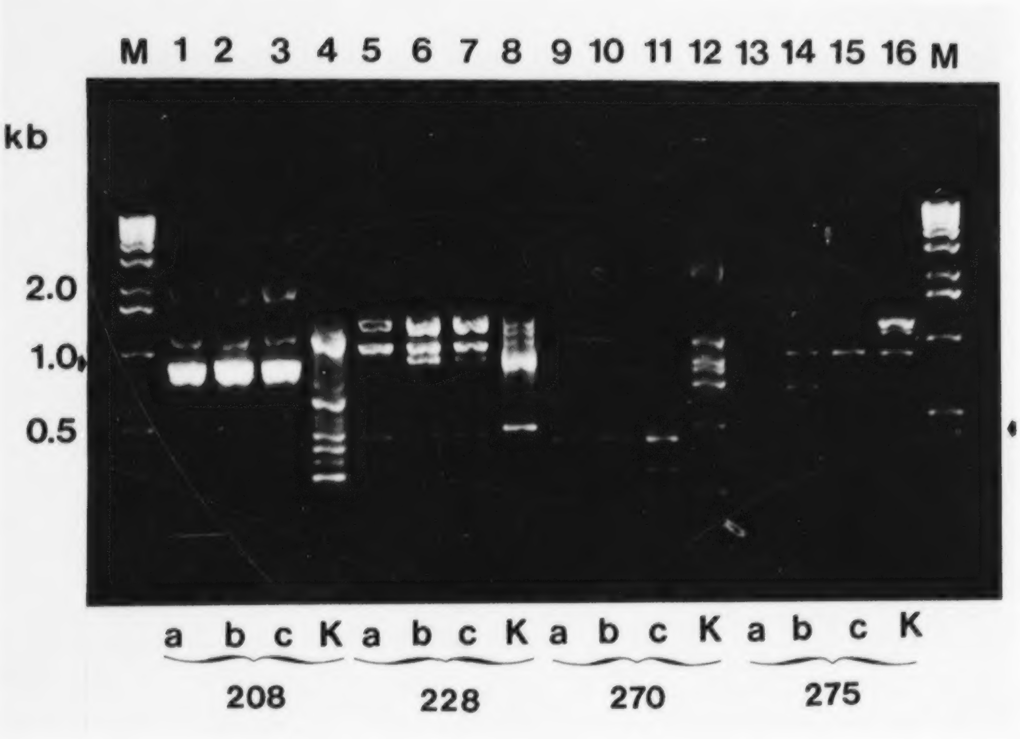

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
kb
2.0
1.0
0.5
a b c K a b c K a b c K a b c K
208
228
270
275

FIGURE IV.2

RAPD primer screen of primers 277, 284, and 287 on pertussis PFGE types a, b, and c and *P. aeruginosa* PAK (labelled K). M: 1 kb molecular weight marker.

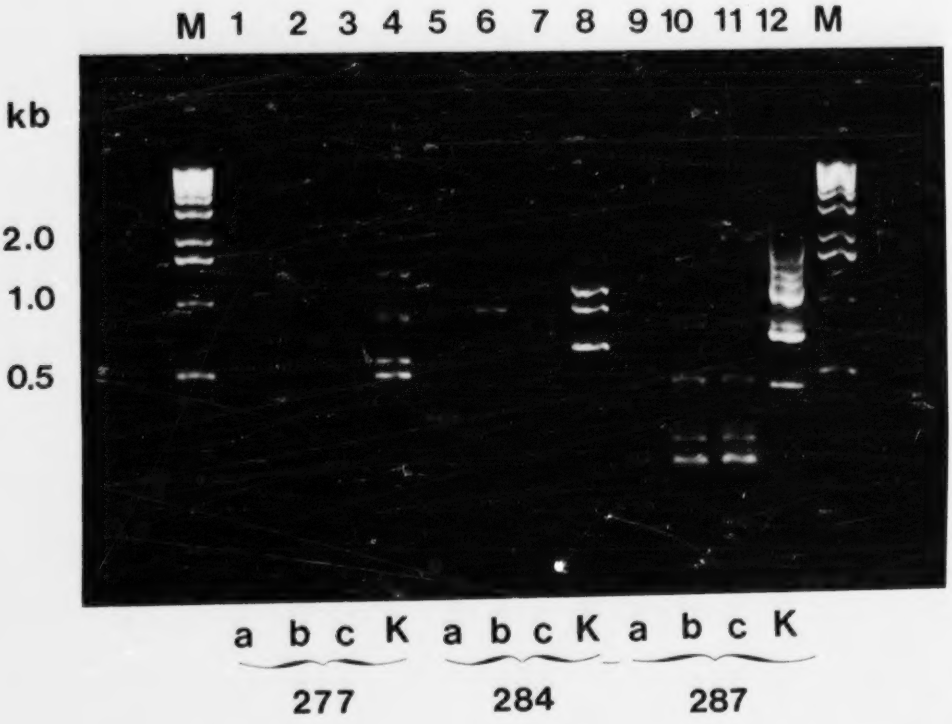
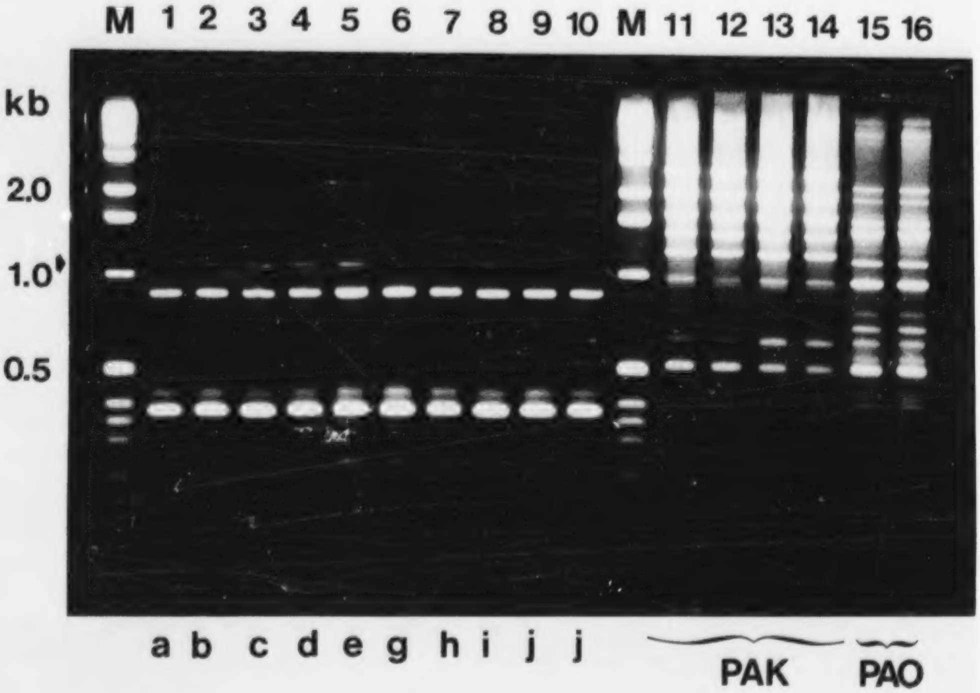

M 1 2 3 4 5 6 7 8 9 10 11 12 M
kb
2.0
1.0
0.5
a b c K a b c K a b c K
277
284
287

FIGURE IV.3

RAPD analysis of pertussis PFGE types a through j (type j in duplicate) along with *P. aeruginosa* strains PAK and PAO with primer 272. M: molecular weight marker. This figure is the result of work done Dr. Eshwar Mahenthiralingam (funded by CBDN) of the University of British Columbia and is included within this thesis with his permission.


M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16
kb
2.0
1.0
0.5
a b c d e g h i j j
PAK
PAO

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CHAPTER V

DISCUSSION

In August 1993, the Canada Communicable Disease Report published the guidelines recommended by the Pertussis Consensus Conference in May of that year (17). Pertussis was ranked as the fifth priority disease for national surveillance by the Advisory Committee on Epidemiology (ACE) (17). Not surprisingly, recommendations on surveillance and epidemiology of *Bordetella pertussis* was therefore one of the top priorities of the consensus conference (17).

The work with pulsed-field gel electrophoresis for pertussis epidemiology as presented in this thesis has been an important contribution to this priority. This methodology has proven to be a sensitive, reproducible means of tracking epidemiologically related strains of pertussis. The major downfall of a PFGE based typing system is that it is very time consuming in comparison to other methods (Table V.1). The advantage of PFGE is that it can be used as a 'reference' typing method for comparison to other molecular typing strategies. Future study into molecular typing methods for pertussis epidemiology will continue because of this. This chapter will review the current molecular typing methods in the context of their application to *B. pertussis* epidemiology and to the pertussis clonality issue. This chapter will also set the framework for future study in this field.

A. MOLECULAR TYPING METHODS FOR PERTUSSIS EPIDEMIOLOGY

The first chapter of this thesis summarized the molecular typing methods currently in use that have been applied to the surveillance of pertussis. The general theme of that chapter consisted of the introduction of a certain method followed by the phrase "This technique has been used to successfully type or trace several bacterial genera with the exception of *B. pertussis*". There were two methods to which this statement did not apply. The first was PFGE and the second was RFLP typing using a probe to the recently discovered IS1002 (12).

Table V.1 is a comparison between the different typing methods reviewed earlier in this thesis. Some methods require little manipulation and genetic types can be determined in as little as 14 hours (RAPD-PCR) whereas others require considerable longer time (120 hours; PFGE). It also summarizes the discriminatory power of each method as being incapable of strain typing (eg: RFLP with a probe IS481) to capable of strain typing (eg: PFGE and RFLP with probe to IS1002).

The ability of RFLP typing pertussis strains using IS1002 as a probe was compared to PFGE typing. IS1002 was discovered by Anneke van der Zee of Dr. Fritz Mooi's lab in the Netherlands (12). We provided them with 15 pertussis strains, PFGE types a through o, so they could analyse them by RFLP. In a personal communication, they reported that, after chromosomal digestion with *Sma*I and hybridization with IS1002, 12 RFLP types could be distinguished. The identical RFLP patterns were observed between types g and j, i and l, and m and n. However, the types a through o strains sent to them also contained the once assumed 'type f' prior to the discovery of the true identity of 'type f'. There was no observation of an identical RFLP pattern between types c and 'f' even though their PFGE types are identical. RFLP analysis using IS1002 as the probe may be the time saving alternative once more strains can be analysed and compared by both techniques.

B. MOLECULAR TYPING AND RELEVANCE TO THE PERTUSSIS CLONALITY ISSUE

Prior to the advent of molecular typing techniques, phenotypic characteristics as defined by serology were used for pertussis strain discrimination. However, serological classification of pertussis is insensitive due to the lack of diverse antigenic determinants. The main targets of serology such as lipooligosaccharide and agglutinogens are not susceptible to change. Phenotypic diversity is based on the presence or absence of agglutinogens 2, and 3 in combination with the always present agglutinogen 1. Thus, *B. pertussis* strains are either one of three major serotypes: 1,0,3; 1,2,0; and 1,2,3.

The pertussis clonality issue was raised as a result of data shown by multilocus enzyme electrophoresis (MEE). Twenty-three strains of *B. pertussis* could be assigned to only three electromorph types (ET), which included the ET unique for the mouse intracerebral challenge strain (13). All 21 *B. parapertussis* strains, could be assigned to only one ET. These data prompted the proposition that the three species within the genus *Bordetella* be considered one species because the population structure was essentially clonal (13).

Recently, this issue has been addressed using other strategies. Restriction fragment length polymorphisms (RFLP), generated simply through the use of frequent cutting restriction endonucleases (4), or in conjunction with a probe to IS481 (7), or to chromosomal genes encoding rRNA (ribotyping) (16), would seem to confirm the clonality of *B. pertussis* strains.

However, if pertussis is as clonal as this research suggests, why does an effective vaccine still elude us? Why does a whole cell vaccine lacking agglutinogen 3 not protect against infection caused by strains of serotype 1,3 (20)? The answer to this may be due to variable degrees of epitope diversity which would not be present in a clonal organism. In combination with the fact that pertussis is strictly a human disease, would the presumed clonality of the organism be the major factor involved in the eradication of *B. pertussis*? Perhaps *B. pertussis* is more diverse than originally thought.

The application of pulsed-field gel electrophoresis (PFGE) to this issue seemed to restore the hope of finding a method to characterize strains such that the prevalence of certain PFGE types might have enough significance to reexamine current vaccine preparations. Appropriately, the first publication of *B. pertussis* epidemiology based on PFGE was entitled 'Is Pertussis Clonal?' (8) and the Matthews' research group showed that this organism has genetic diversity. They also reported that two vaccine strains were the same PFGE type as their most predominant strain (type 1; 22/105 isolates) and speculated as to the efficacy of a vaccine containing these strains (8).

We also obtained convincing evidence of genetic diversity with pertussis strains when isolates from a large

Alberta whooping cough outbreak (6) were analysed by PFGE (3). Our data showed the clustering of genetically homogeneous isolates within family members and within isolated communities and evidence of spread of the organism between a large city (Edmonton) and smaller surrounding northern Alberta towns (3).

Shortly after we published our data, another publication by Matthews' group addressed the clonality of all species of the genus *Bordetella* (9). According to the PFGE data they generated, each species was distinguishable from the other and each species could be further subdivided because of different PFGE types observed. Another important question, concerning the use of the virulent, intracerebral mouse challenge strain 18-323 to assess vaccine efficacy was also addressed by these researchers (10). They discovered this strain exhibited a distinct genetic type from that of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* and they expressed doubts as to the suitability of strain 18-323 for assessing the efficacy of pertussis vaccines (10). On the other hand, they did verify that the 18-323 strains from around the world were genetically identical thus confirming the uniformity of the intracerebral mouse challenge.

In the third chapter of this thesis, the relevance of molecular typing for pertussis and the clonality issue was raised again when minor discordances within our PFGE typing system was observed. Certain strains of the same PFGE type could be made to look different when different enzymes or resolution parameters were employed. These discordances caused us to ask, by what criteria, do we define a strain? This prompted the immunization of mice with either pertussis PFGE type a or type b organisms (courtesy Lisa Purdy) to determine if significantly different immunological responses could be generated. Our results showed that there was more intermouse variability when sera from individual mice were used in Western analysis of type a and type b strains than between the type a and type b strains themselves. For example, mice from one group were immunized with type b organisms. The collected sera was then used in a Western blot of SDS-PAGE resolved type a and type b strains. Each individual mouse could not differentiate type a and type b strains however, the type a and type b pattern pair from the Western analysis using sera from one mouse was different from the type a and type b pattern pair using sera from another mouse from the same group. When the polyclonal sera from group a mice were pooled and sera from group b mice were pooled and subsequently used for Western analysis of PFGE types a through u strains, we observed identical profiles. The pooled sera, however, was not absorbed, thus there were several common antibody clones with both pools to sufficiently mask any potential unique antibody clones that could discriminate between strains. These preliminary findings will be studied further to determine if such unique antibody clones exist.

The existence of two PFGE systems for pertussis epidemiology has also complicated matters. Strains that have been typed as different by one system are typed as identical by the other and vice versa. Again, the criteria by which we and Matthews' group defines a strain is different. Collaborations must be arranged to resolve these issues.

Random-amplified polymorphic DNA (RAPD) is a technique that has been very effective for typing many bacterial genera (1, 2, 11). However, preliminary results with this method have shown it to be unsuitable for *B. pertussis* epidemiology due to the homogeneous profiles that were generated for different, by PFGE, pertussis strains. These results tend to lend support to the notion that pertussis is clonal. However, the question of clonality of different bacterial species is not one that is clearly defined. A paper that questions the clonality of bacteria has shown that some species (e.g.: *Salmonella*) are clonal at all levels of analysis, while the genus *Neisseria* is polyclonal showing diversity at all population levels (18). These two examples represent extreme cases. An example of intermediate population structure was *Neisseria meningitidis* that displays what Smith et al. call an 'epidemic' structure due to significant association between loci (via MEE) due to recent increases in electromorph types (18). However, when the increases in electromorph types are eliminated, *N. meningitidis* becomes as polyclonal as strains in the genus *Neisseria*. This work supports the hypothesis that bacterial clonality exists at all levels.

The comparison of different bacterial typing methods has not been unique to the epidemiologic study of *B. pertussis*. Several of the methods mentioned within this thesis in addition to other techniques have been compared with *Staphylococcus aureus* isolates (19) as well as with *Neisseria gonorrhoeae* isolates (14, 15). On one hand, the DNA-based molecular methods employed were equally very effective for discerning between *S. aureus* isolates except biotyping because too many subtypes were generated (19). On the other hand, the DNA-based molecular methods for *N. gonorrhoeae* had varying degrees of effectiveness. As observed for pertussis isolates, MEE analysis of *N. gonorrhoeae* isolates requiring proline, citrulline, and uracil (PCU-) indicated that PCU- isolates are essentially clonal (15) while ribotyping could only distinguish the 23 isolates into four groups. These same isolates, analysed by PFGE in a subsequent study (14), could be assigned to 11 PFGE patterns. Observations of different PFGE patterns with one enzyme and similar patterns with another enzyme occured as did within chapter III of this thesis. Unlike pertussis, differences between *N. gonorrhoeae* PCU- isolates could be more effectively resolved with the use of a combination of three panels of monoclonal antibodies (14). The authors concluded by stating that the use of any particular method would depend upon the purpose of the study. For example, the use of auxotyping, serotyping and plasmid content would suffice for surveillance of *N. gonorrhoeae* (5) while the use of PFGE and extended serotyping with additional monoclonal antibodies may be useful for subtyping outbreak strains or for assessing their clonality (14).

In conclusion, to say *B. pertussis* is clonal may not be accurate, although this organism is relatively clonal in that there are many phenotypic and genotypic similarities. With further research on this issue, there may be a technique, method, or strategy that may allow us to capitalize on the limited variability of *B. pertussis*. The use of pulsed-field gel electrophoresis to this end has been an encouraging start. The combination of several techniques has been applied with other bacterial genera (14, 15, 19) for epidemiological typing. Perhaps the same can be done for *Bordetella pertussis*.

Table V.I Time Comparison of Molecular Typing Methods for Pertussis Epidemiology*.

Method	Protein /DNA Preparation (hours)	Protein /DNA Manipulation (hours)	Assay Time (hours)	Total Test Time (hours)	Discriminatory Power	Reference
1. MEE	2	8	8	18	Very Little[a]	(13)
2. RFLP						
a) IS481	5	3	24	32	None[b]	(7)
b) IS1002	5	3	24	32	Good[c]	(12)
c) rRNA (ribotyping)	5	3	24	32	Very Little[d]	(16)
3. PFGE	68	24	28	120	Very Good[e]	(3)
4. PCR						
a) LM-PCR	5	7	6	18	Unsuccessful[f]	(4)
b) RAPD	5	1	8	14	Very Little[g]	(4)

Comments:

[a] 23 strains of *B. pertussis* assigned to three electromorph types

[b] IS481 is a conserved insertion sequence (IS) without variability

[c] 12 different RFLP types with 14 different PFGE types

[d] 41 of 42 strains tested had an identical ribotype

[e] 109 clinical strains of *B. pertussis* have been assigned to 20 PFGE types and contact between siblings and individuals from distant communitites has been demonstrated

[f] generation of products was unsuccessful

[g] successful generation of products, however very minor variability seen

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* Times are estimates for the processing of twenty *B. pertussis* clinical isolates and does not include time for growth of the organism.

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